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EFFECTS OF VITAMIN C ON DNA DAMAGE, IRON HOMEOSTASIS AND GENE EXPRESSION PROFILING IN HUMAN FIBROBLASTS

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Vitamin C (ascorbic acid, AA) is an important antioxidant in human plasma. AA has, however, other important, non-antioxidant roles in cells. Of particular interest is its involvement in iron metabolism, since AA enhances dietary iron absorption, promotes Fenton reactions *in vitro* and was reported to have deleterious effects in individuals with iron overload. The mechanisms of AA-driven free radical generation from iron have been the subject of great discussion. However, whilst the AA-driven Fenton reaction is well established in test tube reactions, the relevance of this pro-oxidant chemistry in cells, tissues or organisms remains unclear. To understand whether AA has an overall antioxidant or pro-oxidant effect in cells, the present study investigated its ability to modulate iron homeostasis and iron-mediated oxidative injury in primary normal human diploid fibroblasts. Results show that AA increased the levels of intracellular catalytic iron and concomitantly modulated the expression of two well known iron-regulated genes, ferritin and transferrin receptor. Notably, treatment of confluent fibroblasts with physiologically relevant concentrations of AA was not harmful but sensitised cells towards iron-dependent, H_2O_2-induced DNA strand breakage and cell death. The possibility that AA regulates cellular function, through its effects on the intracellular redox state and/or on iron homeostasis, was also addressed by performing a genome-wide analysis of AA-induced gene expression in human fibroblasts. Results showed that AA stimulates quiescent cell populations to re-enter the cell cycle and proliferate.

In summary, this work shows that AA increases iron availability and enhances ROS-mediated, iron-dependent damage in human cells. It is proposed that AA may exacerbate the deleterious effects of metals *in vivo* and promote normal tissue injury in situations associated with elevated ROS production. It is also suggested that AA may promote fibroblast activation during the process of wound healing, where quiescent dermal fibroblasts are required to readily undergo cell division.

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Contents

Abbreviations

8-OHA	8-hydroxy-2'-adenine
8-OHdA	8-hydroxy-2'-deoxyadenine
8-OHdG	8-hydroxy-2'-deoxyguanine
8-OHG	8-hydroxy-2'-guanine
A	Adenine
AA	Ascorbic acid
AA2P	L-ascorbate 2-phosphate
ActD	Actinomycin D
AFR	Ascorbate free radical
AFU	Arbitrary fluorescence units
ANOVA	Analysis of variance
AP	Apurinic/apyrimidinic
AP-1	Activating protein 1
APE1	AP endonuclease 1
ATP	Adenosine triphosphate
BER	Base excision repair
Bio-NTP	Biotin-labelled ribonucleotide
BIP	2,2'- bipyridyl
BLAST	Basic Local Alignment Search tool
BLM	Bleomycin
bp	base pair
BRCA1	Breast cancer 1
BSA	Bovine serum albumin
C	Cytosine
CA-AM	Calcein acetoxymethylester
CAT	Catalase
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
CDS	Coding domain sequence
CHO	Chinese hamster ovary
CHX	Cycloheximide

cRNA	Complementary ribonucleic acid
Ct	Threshold cycle
CVD	Cardiovascular disease
Da	Dalton
dA	deoxyadenine
dC	deoxycytosine
dChip	DNA-Chip Analyser
DEPC	Diethyl pyrocarbonate
DFO	Desferrioxamine
dG	Deoxyguanine
DHA	Dehydroascorbate
DHLA	Dihydrolipoic acid
DMSO	Dimethylsulphoxide
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleotide triphosphates
DSB	Double-strand breaks
ds-cDNA	Double stranded complementary deoxyribonucleic acid
dT	Deoxythymidine
DTPA	Diethylenetriaminepentaacetate
E. coli	*Escherichia coli*
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Endo III	Endonuclease III
ERCC1	Excision repair cross-complementing 1
ES	Embryonic stem
EST	Expressed sequence tag
EtBr	Ethidium bromide
FAC	Ferric ammonium citrate
FACS	Fluorescence activated cell sorter
FAPY	Formamidopyrimidine
FBS	Foetal bovine serum

FITC	Fluorescein-5-isothiocyanate
FL2-A	Area of the fluorescence peak
FL2-W	Width of the fluorescence peak
FPG	FAPY DNA glycosylase
FSC	Forward-angle light scatter
Ft	Fluorescence threshold
FZN	Ferrozine
G	Guanine
G_0 phase	Quiescent or Gap phase 0
G_1 phase	Gap phase 1
G_2 phase	Gap phase 2
GADD45α	Growth arrest and DNA damage-inducible, alpha
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC/MS	Gas chromatography/mass spectrometry
gdC	Deoxycytidine-glyoxal
GGR	Global genome repair
GLUT	Glucose transporter
GSH	Glutathione
HFE	Haemochromatosis gene
HDF	Human diploid fibroblast
HH	Hereditary haemochromatosis
HO-1	Haem oxygenase-1
HBSS	Hanks' balanced salt solution
HIV	Human immunodeficiency virus
HPLC	High-pressure liquid chromatography
HPLC-EC	High-pressure liquid chromatography with electrochemical detection
HPLC-UV	High-pressure liquid chromatography with ultraviolet detection
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HUVE	Human vascular endothelial
ICAM-1	Intracellular adhesion molecule 1
IRE	Iron-responsive element
IRP	Iron-regulated protein
IVT	*In vitro* transcription

Keap1	Kelch-like ECH-associated protein 1
LA	Lipoic acid
LDL	Low-density lipoprotein
LET	Linear energy transfer
LIP	Labile iron pool
LMP	Low melting point
M phase	Mitosis phase
MDA	Malondialdehyde
MEM	Minimal essential medium
MES	2-Morpholinoethanesulfonic acid
MLH1	Mut L homologue-1
MMR	Mismatch repair
MOPS	4-Morpholinepropanesulfonic acid
MPA	Metaphosphoric adic
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADPH	Nicotinamide-adenine dinucleotide phosphate, reduced
NCBI	National Centre for Biotechnology Information
NER	Nucleotide excision repair
NO	Nitric oxide
Nrf2	NF-E2-related factor 2
NTBI	Non-transferrin-bound iron
O.D.	Optical density
PARP1	Poly(ADP-ribose) polymerase 1
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PG SK	Phen Green SK
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
QPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease

ROS	Reactive oxygen species
RPM	Rotations per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
S phase	Synthesis phase
SAPE	Streptavidin-phycoerythrin
SCGE	Single-cell gel electrophoresis
S.D.	Standard deviation
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
SSB	Single-strand breaks
SSC	Side-angle light scatter
SSPE	Sodium Chloride/Sodium Phosphate/EDTA
T	Thymine
T7	Bacteriophage T7
TCR	Transcription-coupled repair
TFRC	Transferrin receptor
Tm	Melting temperature
T-RNA	Total ribonucleic acid
U	Uracil
UTR	Untranslated region
UV	Ultraviolet
VSMC	Vascular smooth muscle cell
XP	Xeroderma pigmentosum

CHAPTER I

INTRODUCTION

1.1. Oxidative Stress

1.1.1. The concept of oxidative stress

The reduction of molecular oxygen to water is the major source of energy for most aerobic organisms. Molecular oxygen at physiological concentrations by itself and water are not very reactive. However, when partially reduced, they produce more reactive oxygen species (ROS) that can be detrimental to cells by damaging biological molecules (lipids, proteins and DNA). The production of ROS can, however, be balanced by the existence of cellular antioxidant defences, including enzymes that remove ROS (*e.g.* superoxide dismutase, catalase, peroxidase), proteins that sequester transition metal ions (ferritin, transferrin), low molecular weight peptides and cofactors (*e.g.* glutathione, thioredoxin, reduced nicotinamide-adenine dinucleotide phosphate or NADPH) and lipid- and water-soluble low molecular weight dietary agents that scavenge reactive oxygen and nitrogen species (*e.g.* vitamin E, vitamin C and β-carotene). In fact, ROS are involved in normal biochemical processes, including the control of cell proliferation (Burdon 1994), cell signalling (Finkel 1998), and the anti-microbial action of phagocytic cells (Forman and Thomas 1986). However, an imbalance in the cellular redox state, where the production of ROS overwhelms the cellular antioxidant capacity, results in the condition termed *oxidative stress*.

Oxidative stress is thought to be involved in the aetiology of a wide variety of diseases, including atherosclerosis, diabetes, neurodegenerative diseases, chronic inflammatory diseases, cancer and in ageing (Cross *et al.* 1987; Halliwell and Gutteridge 1999; Schumacker 2006). The sources of ROS, the cellular antioxidant defences, and the effects of ROS in cells and organisms are illustrated in Figure 1.1.

1.1.2. Mechanisms of ROS generation in living cells

The term ROS includes oxygen free radicals, *i.e.* oxygen-derived species capable of independent existence that contain one or more unpaired electrons, like the superoxide anion ($O_2^{\bullet -}$), the hydroxyl radical ($HO^{\bullet}$), the alkoxyl radical ($RO^{\bullet}$), the alkyldioxyl radical ($ROO^{\bullet}$) and the oxides of nitrogen ($NO^{\bullet}$, $NOO^{\bullet}$). The term ROS also includes non-radical intermediates such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and singlet oxygen (Farber 1994; Halliwell and Cross 1994).

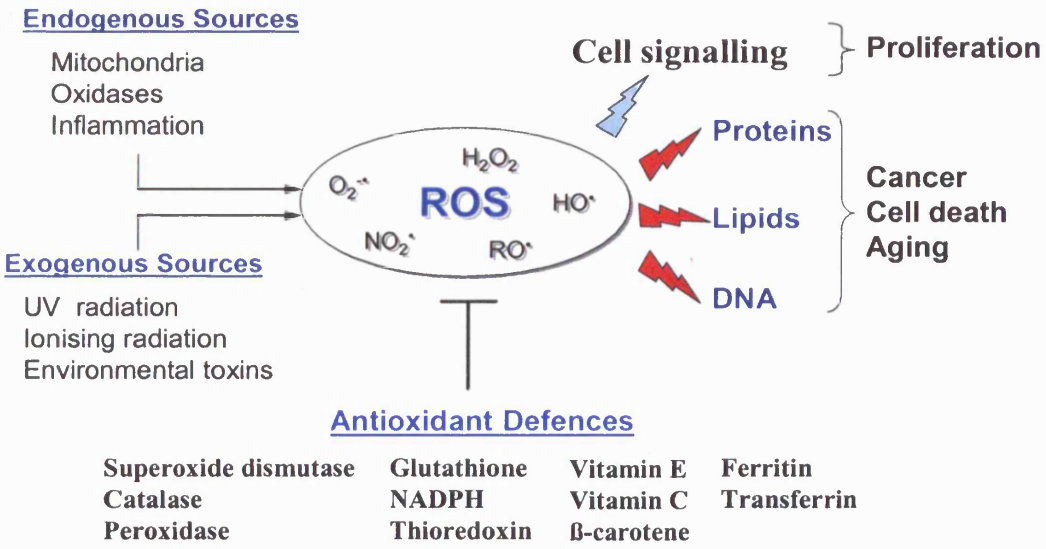


Figure 1.1. The formation, removal and effects of reactive oxygen species (ROS) in cells and organisms.

The diatomic oxygen molecule (O_2) is itself a radical, having two unpaired electrons with parallel spin, each in a π^* anti-bonding orbital. So, for O_2 to oxidise a molecule, the two incoming electrons would have to be of parallel spin to fill the vacant spaces in the π^* orbitals. However, most biomolecules are covalently bonded non-radicals, with the two electrons in a covalent bond having anti-parallel spins and occupying the same orbital. For this reason, the reaction of molecular oxygen with biomolecules is spin-forbidden (reviewed by Halliwell and Gutteridge 1990). Many transition metals, however, can overcome the spin restriction of oxygen due to their ability to accept and donate single electrons, thus enhancing the rates of biomolecule oxidation. In this respect, transition metals may simultaneously bind biomolecules and oxygen, serving as a bridge between the two (Miller *et al.* 1990).

Another way of increasing the reactivity of oxygen is to move one unpaired electron in a way that alleviates the spin restriction, thereby generating singlet oxygen ($^1O_2$). Singlet oxygen may exist in two possible states, one with no unpaired electrons and one with the two unpaired electrons still in separate orbitals but with anti-parallel spins. The latter is very unstable and therefore only the former is important in biological systems. The conversion of oxygen to singlet oxygen requires energy. Typically, this occurs when a

pigment is excited in the presence of oxygen. The pigment absorbs light and enters a higher electronic excitation state, then transfers the energy to the oxygen molecule to yield singlet oxygen. The reactions of biomolecules with singlet oxygen are not spin-restricted and therefore singlet oxygen reacts with many biomolecules as an electrophile to form peroxides (reviewed by Halliwell and Gutteridge 1990).

ROS can be generated endogenously as a result of normal intracellular metabolism or due to the activity of phagocytic cells at sites of inflammation. The endogenous generation of oxygen-derived reactive species occurs mostly in the mitochondria and has been extensively reviewed (Halliwell and Gutteridge 1990; Chaudière 1994; Farber 1994; Halliwell and Cross 1994; Koppenol 1994; Finkel and Holbrook 2000). Briefly, small quantities of superoxide anion, the product of the one-electron reduction of oxygen, are constantly generated due to "electron leakage" resulting from the auto-oxidation of the respiratory chain components, in the inner mitochondrial membrane. Although these components usually pass the bulk of electrons onto the next component of the respiratory chain, some seem to "leak" electrons to oxygen. Hence, all aerobic cells have a constitutive flux of superoxide anion formation, resulting from the one-electron reduction of oxygen. Superoxide anion can also be produced by the auto-oxidation of metal complexes or from several compounds that undergo redox cycling inside the cells, like many semiquinones ($SQ^{\bullet -}$):

$$SQ^{\bullet -} + O_2 \longrightarrow \text{Quinone} + O_2^{\bullet -} \quad [1]$$

In addition, superoxide is produced by enzymes (oxidases) that oxidise their substrates and reduce oxygen, such as during the respiratory burst of phagocytic cells (neutrophils, monocytes and macrophages). Upon stimulation, phagocytic cells undergo a respiratory burst characterized by a marked increase in glucose metabolism and oxygen consumption, which leads to the release of superoxide, H_2O_2 and hypochlorous acid into the surrounding tissues. The source of superoxide generation is a membrane-associated oxidase, which transfers electrons from NADPH to oxygen. Other major components of the system include a b cytochrome and a flavoprotein.

$$2O_2 + \text{NADPH} \longrightarrow 2\ O_2^{\bullet -} + \text{NADP}^+ + H^+ \quad [2]$$

Hypochlorous acid is generated from H_2O_2 in a process mediated by the phagosomal myeloperoxidase present in stimulated neutrophils. The production of ROS that can

damage cellular components during the respiratory burst is essential for the bactericidal activity of neutrophils (Forman and Thomas 1986).

Superoxide is only moderately reactive in solution, acting mainly as a reducing agent. It is unstable and decomposes either spontaneously or in the presence of superoxide dismutases (SOD), at a much faster rate. Thus SODs remove superoxide anion by accelerating the spontaneous dismutation of superoxide, yielding oxygen and H_2O_2:

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad [3]$$

Consequently, a system that produces superoxide is also expected to produce H_2O_2 by dismutation. Indeed, H_2O_2 is also produced in the mitochondria and during the respiratory burst of phagocytic cells. H_2O_2 has no unpaired electrons and therefore is not a radical. On its own, H_2O_2 has poor reactivity but it can readily cross biological membranes. The addition of one electron to the oxygen-oxygen bond of H_2O_2 by oxidation of ferrous iron (Fe^{2+}) results in the formation of hydroxyl radical and the accompanying formation of ferric iron (Fe^{3+}). This reaction is also catalysed by the cuprous ion and is known as the Fenton reaction (see Section 1.3.3 for more information):

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO^{\bullet} \quad [4]$$

In a similar way, the reaction between metal ions and alkyl hydroperoxides (ROOH) leads to the formation of the alkoxyl radical ($RO^{\bullet}$):

$$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + OH^- + RO^{\bullet} \quad [5]$$

The alkyldioxyl radical ($ROO^{\bullet}$) is formed from an alkyl radical and oxygen, without mediation by a metal complex (Koppenol 1994).

The hydroxyl, the alkoxyl and the alkyldioxyl radicals are all oxidising species. The hydroxyl radical, which is also produced when water is exposed to high-energy ionising radiation, is highly unstable, and will react with most biologic molecules, being the most reactive of all the oxygen radicals. It is believed that most of the toxicity of superoxide anion and H_2O_2 involves their conversion into hydroxyl radical (Farber 1994; Halliwell and Cross 1994).

1.1.3. Mechanisms of cell injury by ROS

The mechanisms of cell injury by ROS have been extensively studied and reviewed (Farber 1994; Halliwell and Cross 1994; Marnett 2000). Oxidative stress damages cellular

proteins, lipids and DNA and is implicated in carcinogenesis, cell death and ageing (reviewed by Halliwell and Cross 1994; Wang *et al.* 1998; Finkel and Holbrook 2000). ROS attack on DNA induces a plethora of DNA base modifications, sugar moiety damage, strand breaks, base-free sites, inter-strand/intra-strand crosslinks and DNA-protein crosslinks. The mechanisms leading to oxidative damage to DNA have been extensively reviewed (Wang *et al.* 1998; Evans *et al.* 2004). Oxygen radicals can modify DNA bases covalently causing many different DNA lesions (or adducts) in each of the four bases. The hydroxyl radical is a highly reactive molecule that acts by addition or abstraction. It adds to double bonds of heterocyclic DNA bases and abstracts an H atom from the methyl group of thymine and from each of the C-H bonds of 2'-deoxyribose. The resulting C- or N-centred radicals of DNA bases and C-centred radicals of the sugar moiety can be further reduced or oxidised, yielding a variety of final products. For example, cytosine and thymine glycol can be formed from the C5-OH adduct radicals of cytosine and thymine, respectively, while 5-hydroxy-5-methylhydantoin can be formed from the C6-OH-adduct radical of thymine, and the allyl radical that results from H-abstraction from thymine yields 5-hydroxymethyluracil. The C8-OH-adduct radical of guanine or adenine can undergo either one-electron oxidation or reduction (with ring opening) to yield 8-hydroxypurines or formamidopyrimidines, respectively.

Some of these lesions cause base mispairing, leading to transitions/transversions and hence may be premutagenic (Wang *et al.* 1998). The most extensively analysed form of oxidative DNA damage is the product of guanine oxidation at position C-8, 8-hydroxy-2'-deoxyguanosine (8-OHdG). The 8-OHdG lesion is considered to be a useful marker of oxidative stress. It is mutagenic, causing mispairs with dA and consequently leading to GC $\rightarrow$ TA transversions (Kasai 1997). However, basal levels of 8-OHdG in the genome remain controversial due to discrepancies between the different methods used for detection, and to the artifactual oxidation of guanine prior to analysis (Kasai 1997), as described in Section 1.4.6.

Hydroxyl radical attack on the sugar moiety of DNA also leads to the formation of C-centred radicals. These radicals can undergo a one-electron oxidation and subsequently react with water, resulting in the loss of the base to yield a 2-deoxypentos-4-ulose moiety within the DNA. Additionally, this radical can lose the phosphate group on either side of

the DNA chain, yielding strand breaks and two different radical cations as end groups. Additional sugar modifications can result from reaction of the sugar radical with oxygen. In addition to modified sugars, modified DNA bases can be lost due to the weakening of the glycosidic bond, yielding base-free or apurinic/apyrimidinic (AP) sites. Finally, a DNA base radical can be added to an aromatic amino acid of proteins or combined with an amino acid radical to yield a covalent DNA–protein cross-link (Evans *et al.* 2004).

Potent oxidising species (including the hydroxyl radical) can also attack polyunsaturated fatty acid residues of membrane phospholipids, by abstracting a hydrogen atom from a reactive methylene (—CH_2—) group and thereby forming a lipid radical ($L^{\bullet}$) that reacts with molecular oxygen to form lipid peroxyl radical ($LOO^{\bullet}$). This radical is itself a moderately strong oxidising species and will extract a new hydrogen atom from a neighbouring polyunsaturated lipid, initiating a new free radical attack. These propagation reactions can be repeated many times and lead to the formation of lipid hydroperoxides (LOOH). This autocatalytic chain reaction is named lipid peroxidation and will gradually destroy the unsaturated fatty acids of membrane phospholipids and alter ion transport and membrane permeability (see reviews in Rice-Evans 1994; Farber 1994). Additionally, the resulting lipid hydroperoxides are unstable and decompose to very reactive intermediates, like malondialdehyde (MDA) and many others, which are able to modify DNA bases and form adducts (Marnett 2000).

Oxidative stress can also damage the proteins involved in calcium and sodium homeostasis and iron storage, thereby increasing the intracellular levels of Ca^{2+} and free iron, further causing cell injury or cell death (Chaudière 1994; Farber 1994; Halliwell and Cross 1994).

1.1.4. Exogenous sources of ROS

Cell injury can also occur as a result of ROS production from exogenous sources such as ultraviolet radiation, ionising radiation and the metabolism of redox cycling drugs and environmental toxins (*e.g.* cigarette smoke, paraquat) (Halliwell and Cross 1994; Finkel and Holbrook 2000).

Ionising radiation causes changes in the cells or tissues through which it passes by depositing energy and inducing ionisation and excitation of the molecules with which it

interacts. Different radiation types deposit their energy at different rates, the rate being characterised as the linear energy transfer (LET). Alpha particles, neutrons and iron ions are densely ionising and therefore are high LET, whereas X-rays and gamma-rays are low LET ionising radiation (Ward 1988; Smith *et al.* 2003).

Radiation damages the DNA either by direct energy deposition in the DNA molecule or through indirect attack on the DNA molecule of reactive species generated by ionisations in other molecules. As water is the most abundant molecule *in vivo*, the hydroxyl radicals generated from water radiolysis are the main source of this indirect effect. The initial damage is randomly distributed among the DNA bases and the deoxyribose moieties. DNA lesions include single- and double-strand breaks, sugar damage, base damage, AP sites and DNA-protein crosslinks. Due to the nature of the energy deposition events (*i.e.* formation of several reactive species that will react rapidly and close to their point of origin), damage can occur as either singly or locally multiple damaged sites. The latter are more difficult to repair and have the potential to become lethal to the cell (Ward 1988). DNA double-strand breaks (DSB) correlate well with cell death induced by ionising radiation and are considered to be the cause of radiation-induced cytotoxicity and mutagenesis (Ward 1995). Even though mammalian cells are able to rejoin most DNA DSB with a fast repair kinetics, the persistence of residual un-rejoined DNA DSB can lead to chromosome aberration and loss of genetic material, and consequently to cell death. It has been argued that cells with slower repair kinetics of DNA DSB are generally more sensitive to ionising radiation, perhaps due to lower fidelity of rejoining (reviewed by Nunez *et al.* 1996; Dikomey *et al.* 1998). Radiation is a well known inducer of genomic instability in mammalian cells, causing large-scale chromosomal rearrangements and aberrations, amplification of genetic material, aneuploidy, micronucleus formation, microsatellite instability and gene mutation, ultimately leading to carcinogenesis (reviewed by Smith *et al.* 2003).

Solar radiation is considered to be a genotoxic agent and most of its cytotoxicity and mutagenicity are associated with the ultraviolet (UV) region of the spectrum (Figure 1.2). UV radiation has sublethal (inhibition of growth and colony-forming ability) and lethal effects (death, mutagenesis and carcinogenesis) on cells (Tyrrell 1994). In fact, exposure to the UV components of sunlight has been extensively associated with skin

ageing and photocarcinogenesis (Trautinger 2001). UV radiation is involved in the formation of a plethora of benign, inflammatory, precancerous and malignant lesions in epithelial (*e.g.* photodermatitis, squamous cell carcinoma), dermal (*e.g.* fibrosarcoma), adnexal (*e.g.* basal cell carcinoma) and pigment cells (*e.g.* melanoma) (reviewed by Stenback 1982).

The two UV components of sunlight that reach the Earth's surface are UVA (320-400 nm) and UVB (280-320 nm), while UVC (200-280 nm) is not biologically relevant in terms of sun exposure. UVB and at a lower extent UVA react with DNA directly through absorption, and indirectly through the generation of reactive oxygen species. The main photoreactions of solar UV radiation within cellular DNA involve the direct absorption of UVB photons by the pyrimidine constituents of DNA bases and the subsequent formation of dimeric pyrimidine photoproducts. The most frequent UV-induced DNA lesions are cyclobutane pyrimidine dimers, followed by the 6-4 pyrimidine-pyrimidone photoproducts. These lesions are the proposed cause of most of the deleterious effects of UV radiation on DNA. Minor effects of UV radiation include DNA strand breaks, DNA-protein crosslinks and oxidation of the guanine residues of DNA (Ravanat *et al.* 2001). Exposure of skin cells to UVA and near visible radiation leads to the formation of singlet oxygen due to the excitation of cellular chromophores or photosensitisers (*e.g.* flavins, prophyrins) and eventually results in oxidative DNA damage (8-OHdG, DNA strand breaks) (Kvam and Tyrrell 1997).

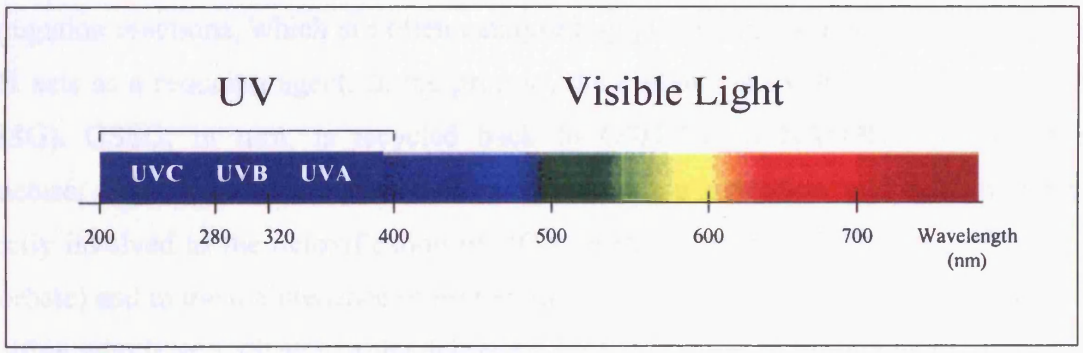


Figure 1.2. Solar spectrum including ultraviolet (UV) radiation and visible light.

1.1.5. Cellular antioxidant defences

Cells can resist the insult of oxidative stress due to the existence of a number of protective mechanisms that intercept ROS and thus prevent oxidative damage, including both enzymatic and non-enzymatic systems (Halliwell and Cross 1994). Antioxidant compounds are amongst the non-enzymatic mechanisms used by organisms to protect against ROS. According to the definition of Halliwell and Gutteridge (1999), *antioxidants are substances that, when present at low concentrations compared to those of an oxidisable substrate, considerably delay or prevent oxidation of that substrate.* A compound may show antioxidant properties by inhibiting production of ROS, by directly scavenging them or by increasing the levels of antioxidant defences in the organism (Halliwell 2002). Amongst the first are proteins involved in the sequestration of metal ions, especially iron and copper ions, into forms that will not catalyse free radical reactions, as described later (Section 1.3). An important mechanism that protects organisms against ROS is the presence of endogenous reducing agents and free radical scavenger compounds that enter the redox reactions and neutralise reactive species into non-reactive ones by contributing an electron (Rose and Bode 1993). Glutathione (GSH) is one of the most important antioxidant molecules in biological systems. Although it is available from fresh plant and animal products in the diet, most of the GSH found in cells is synthesised by a pathway involving the cytoplasmic enzymes γ-glutamylcysteine and glutathione synthase. GSH is a tripeptide composed of the amino acids glutamate, cysteine and glycine. The thiol group (-SH) of the cysteine residue is the functional portion of the molecule. It reacts with electrophiles in conjugation reactions, which are often catalysed by glutathione *S*-transferases. In addition, GSH acts as a reducing agent. In the process, the cysteine is oxidised to form a disulfide (GSSG). GSSG, in turn, is recycled back to GSH by an NADPH-dependent GSSG reductase. The GSH/GSSG pool undergoes reversible oxidation and reduction and is directly involved in the detoxification of ROS, in the recycling of other antioxidants (*e.g.* ascorbate) and in the maintenance of protein thiols in the reduced state. Therefore, the thiol-disulfide ratio is an indicator of the degree of oxidative stress in plasma and in cells (Jones 2002).

Other examples of radical scavenger molecules are ascorbate (vitamin C), α-tocopherol (vitamin E) and β-carotene (Chaudière 1994; Diplock 1994). Ascorbate acts as

an electron donor at physiological pH and reacts with most hydrophilic free radicals. The antioxidant properties of ascorbate are described in detail in Section 1.4. Vitamin E stands for a group of naturally occurring compounds, α-, β-, γ- and δ-tocopherols and tocotrienols, of which α-tocopherol has the highest biological activity and is the only one that can reverse deficiency symptoms in humans. Vitamin E is thought to be the main lipid-soluble antioxidant defence, protecting unsaturated fatty acids in cell membranes from lipid peroxidation. α- tocopherol interrupts the lipid peroxidation chain reaction by scavenging peroxyl radicals, being the most important scavenger within membranes. Numerous studies have shown that vitamin E can protect against LDL oxidation and atherosclerosis and may help reduce the risk of coronary heart disease (Landvik *et al.* 2002). Carotenoids are a group of compounds with different structure and biological functions, including vitamin A precursors (α-carotene, β-carotene and β-cryptoxanthin). They share a basic molecular structure that includes a long carbon chain with a series of conjugated double bonds in the central part of the molecule, which allows delocalization of electrons along the polyene chain. Due to this property, carotenoids are able to quench singlet oxygen and scavenge free radicals (Deming *et al.* 2002).

Lipoic acid (LA) is a naturally-occurring molecule that is present as a co-factor in several mitochondrial enzymes involved in oxidative metabolism. LA is rapidly transported into cells, where it is reduced to dihydrolipoic acid (DHLA). The antioxidant properties of LA are associated with its reduced form. DHLA is an efficient scavenger of peroxyl radicals and recycles ascorbate, which indirectly leads to the reduction of vitamin E radicals. Hence, DHLA acts as a chain-breaking antioxidant and enhances the antioxidant potency of other important antioxidant molecules (Tirosh *et al.* 2002).

Dietary polyphenols also have antioxidant properties. They include phenolic acids, phenolic polymers (tannins) and flavonoids. Flavonoids are the largest group of plant phenols. Their molecular structure consists of an aromatic ring condensed to a heterocyclic ring and attached to a second aromatic ring. Their antioxidant activity is due to the existence of several phenolic hydroxyl groups attached to the aromatic rings. Polyphenols and flavonoids can exert antioxidant activity by acting as reducing agents, donating hydrogen atoms or scavenging free radicals. In addition, some polyphenols are able to chelate transition metal ions (Fuhrman and Aviram 2002).

Melatonin is a lipid- and water-soluble indole produced in several tissues that is capable of scavenging a number of reactive oxygen and nitrogen species (Livrea *et al.* 2002). Accordingly, melatonin is protective in experimental models of ischemia-reperfusion injury, neurodegenerative diseases, acute local inflammation, lipid peroxidation and against ionizing radiation (Reiter *et al.* 2002).

Selenium is an essential trace element that can be obtained from plant and animal food. Although selenium compounds do not have the properties of direct antioxidants, selenium is a component of proteins that promote the detoxification of several ROS. Examples of these selenoproteins are cytosolic glutathione peroxidases and thioredoxin reductase (Brigelius-Flohe *et al.* 2002).

Nitric oxide ($NO^{\bullet}$) is an endogenously synthesised free radical that mediates vasodilation and neurotransmission, inhibits platelet adherence and aggregation, and is involved in the killing of pathogens by macrophages and neutrophils. Despite being a free radical, nitric oxide is only moderately reactive. It can diffuse through biological membranes and accumulate in hydrophobic areas. Nitric oxide inhibits metal-catalysed oxidation reactions by forming stable metal-nitrosyl complexes. In addition, nitric oxide inhibits lipid peroxidation by scavenging alkoxyl and peroxyl radicals (Rubbo and Radi 2002).

Enzymatic antioxidant mechanisms promote the removal of excessive superoxide anion and H_2O_2. As mentioned above, SODs remove superoxide anion by converting it into oxygen and H_2O_2 (according to equation 3). There are two SODs in the cell: SOD-1 is a Cu,Zn-dependent SOD and is localised in the cytosol, whereas SOD-2, a Mn-dependent SOD, is localised in the mitochondria. Catalase (CAT), in turn, catalyses the two-electron dismutation of H_2O_2 into oxygen and water (equation 6), while glutathione peroxidase, using GSH, reduces H_2O_2 to water (equation 7) and hydroperoxides to alcohols (equation 8) (Chaudière 1994):

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \qquad [6]$$

$$H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG \qquad [7]$$

$$ROOH + 2GSH \longrightarrow ROH + H_2O + GSSG \qquad [8]$$

Subsequently, the enzyme glutathione reductase catalyses the reduction of GSSG by NADPH, thereby recycling GSH:

$$NADPH + H^+ + GSSG \longrightarrow 2GSH + NADP^+ \quad [9]$$

Despite the existence of antioxidant defences, ROS are present in cells, where they have useful metabolic roles, as mentioned above. Hence, the antioxidant defences need to exist in balance with the production of oxygen-species *in vivo*, and consequently cells have a continuous low-level of oxidative damage (Halliwell and Cross 1994; Finkel and Holbrook 2000). For this reason, cells have developed repair mechanisms able to deal with oxidatively damaged molecules, which include repair enzymes that remove DNA damage.

1.2. Cellular responses to stress

As described, cells are continuously exposed to DNA damaging agents arising both from endogenous and exogenous sources. These agents induce modifications in the DNA structure that, if not dealt with, threaten genomic integrity. Eukaryotic cells eliminate or cope with the damage in the following possible ways: (a) activation of a DNA damage checkpoint, which arrests cell cycle progression to allow for repair and prevention of the replication of damaged chromosomes; (b) repair of DNA damage to restore the integrity of the DNA molecule; (c) induction of a transcriptional response, which results in the expression of stress response proteins that may be beneficial to the cell; and (d) activation of programmed cell death or apoptosis, which eliminates heavily damaged or seriously deregulated cells (Sancar *et al.* 2004).

1.2.1. The eukaryotic cell cycle

The cell cycle is the orderly process by which a cell divides into two daughter cells. The eukaryotic cell cycle is a very tightly regulated event that is divided into four phases: a 'gap' (G_1) phase in which the cells prepares for DNA synthesis; a DNA synthesis (S) phase during which the cell replicates the DNA; a second 'gap' (G_2) phase where the cell duplicates its contents to prepare for mitosis; and the phase during which the cell divides in two, the mitotic (M) phase. However, in the absence of mitogenic signals, in the presence of anti-mitogenic factors or as a consequence of contact inhibition, cells withdraw from the cell cycle and enter a stationary or quiescent state (G_0 phase). In fact, most of the normal cells in the body are found in this state. In addition to the nutrients essential for cell growth, external signals (growth factors) are required for cells to switch from G_0 to G_1 and restart

the cell cycle (*e.g.* during wound healing). Growth factors bind to extracellular receptors and elicit a signal-cascade of gene activation that will culminate in the start of DNA synthesis. Beyond a certain point at the end of G_1 phase (the restriction point), however, the cell cycle progression does not depend on external signals anymore and is exclusively governed by a genetic network that is subject to internal controls. The key mechanism regulating transition throughout all the phases of the cell cycle is the formation of complexes between proteins called cyclins and their respective cyclin-dependent kinases (CDKs). Cyclins are the primary modulators of CDK activity. The activity of the cyclin-CDK complexes is controlled by various mechanisms, including CDK phosphorylation/dephosphorylation, phase-specific expression and proteolysis of cyclins, and the binding of inhibitory proteins to the complex (Park and Koff 1998; Pardee 2002; Rajewsky and Muller 2002).

The G_1 phase is generally the longest phase of the cell cycle (6–24h in cultured cells). In early to mid G_1 phase there is an accumulation of cyclin D, which associates with CDK4/6 and triggers the synthesis of cyclin E in late G1 phase. Cyclin E, in turn, activates CDK2, leading to cyclin A expression and DNA synthesis. In early S phase there is an increase in the levels of proteins involved in DNA replication and cyclins D and E are degraded by proteasomes. Cyclin A forms a complex with CDK2 that determines the progression through S phase. This phase usually lasts for 6 to 8 hours, at the end of which the cell has doubled its DNA content. Once DNA synthesis is completed, cells enter the G_2 phase of the cell cycle. This phase takes several hours, allowing for the duplication of the cell contents in preparation for mitosis. Cyclin B starts to accumulate during the S-phase and its expression increases throughout G_2. It binds to a specific CDK kinase, cell division cycle 2 (CDC2), but the complex only becomes active at the G_2/M boundary. In fact, this complex is located in the cytoplasm during G_2 and only at the G_2/M boundary it is rapidly relocated to the nucleus, where it will be responsible for the breaking down of the nuclear membrane. The process of mitosis takes approximately 1 hour to be completed and involves the condensing and pairing of the duplicate chromosomes (prophase), the formation of the mitotic spindle (metaphase), the equal segregation of the chromosomes into the two daughter cells (anaphase) and finally their complete separation (telophase).

During this process, cyclin B and anaphase inhibitors will be degraded prior to the completion of cell division (Park and Koff 1998; Pardee 2002).

1.2.2. DNA damage checkpoints

The progression through the phases of the cell cycle is carefully regulated by internal controls or checkpoints. These checkpoints are biochemical pathways that preserve the accurate transmission of genetic information by monitoring cell cycle events like DNA replication and spindle assembly, and sending signals for the cell machinery to halt cell cycle progression until the detected error is dealt with. These checkpoints control the G_1/S and G_2/M transition points, as well as S phase progression and they can be activated by DNA damage (Figure 1.3). The proteins involved in the DNA damage checkpoint responses are the same as those responsible for the normal progression of the cell cycle, so these are not unique pathways activated by DNA damage but rather pathways that are amplified in response to DNA damage. The DNA damage checkpoints employ damage sensor proteins (*e.g.* ATM, ATR and the Rad17-RCF and Rad9-Rad1-Hus1 complex) to detect DNA damage and initiate signal transduction cascades, resulting in the activation of 'checkpoint' kinases (Chk1 and Chk2) and Cdc25 phosphatases. The signal transducers activate p53 and inactivate cyclin-dependent kinases to inhibit cell cycle progression from G_1 to S, DNA replication or G_2 to mitosis. Even though the checkpoints are distinct, some of the proteins involved in the process are the same (Sancar *et al.* 2004). The tumour suppressor protein p53, for example, is rapidly activated and induces the expression of genes which can cause cells to arrest division at different phases of the cell cycle. A number of CDK inhibitors are able to bind to the cyclin-CDK complex and halt cell cycle progression during the different phases of the cell cycle. One such protein is cyclin-dependent kinase inhibitor 1A (p21), which is induced by p53 under several growth arresting conditions and plays an important role in the cellular response to DNA damage and other types of stress. p21 is an important mediator of the G_1/S transition checkpoint, which prevents cells from commencing DNA synthesis if the presence of DNA damage is detected. The cell cycle arrest then allows for the DNA damage to be repaired (Pardee 2002; Rajewsky and Muller 2002; Sancar *et al.* 2004).

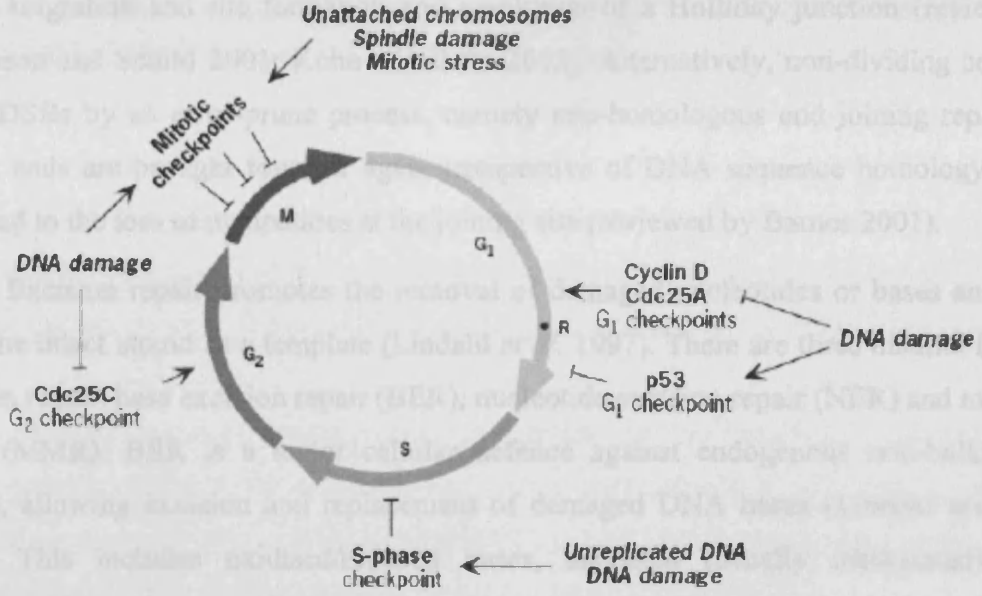


Figure 1.3. Overview of mammalian cell cycle checkpoints (Rajewsky and Muller 2002).

1.2.3. DNA repair

The ability to recognize and repair DNA damage is a common feature to all living organisms (Eisen and Hanawalt 1999). In humans, at least 130 genes are thought to be involved in DNA repair (Wood et al. 2001), and repair processes can be divided into four classes: direct repair, recombinational repair, non-homologous end joining repair and excision repair.

Direct repair is the simplest form of DNA repair and it involves a direct reversal of the adducted DNA to the original chemistry of the base (Josephy 1997). An example is the activity of O^6-methylguanine DNA methyltransferase, which repairs guanine residues with alkyl groups in the O-6 position that can result from chemical carcinogens like nitrosamines and chemotherapeutic agents such as nitrosoureas. The mechanism consists of the transfer of the alkyl group to a cysteine residue in the enzyme active site.

The homologous recombinational repair provides a mechanism for error-free removal of damage (mainly the cytotoxic DSBs and interstrand crosslinks) present in DNA that has replicated before the cell division occurs, by acting in coordination with the S and G_2 checkpoint machinery. The damage is repaired by homologous recombination, using the homologous intact molecule as a template. The process has three steps: strand invasion,

branch migration and the formation and resolution of a Holliday junction (reviewed by Thompson and Schild 2001; Kohn and Bohr 2002). Alternatively, non-dividing cells may repair DSBs by an error-prone process, namely non-homologous end joining repair. The broken ends are brought together again irrespective of DNA sequence homology, which may lead to the loss of nucleotides at the joining site (reviewed by Barnes 2001).

Excision repair promotes the removal of damaged nucleotides or bases and repair using the intact strand as a template (Lindahl *et al.* 1997). There are three distinct forms of excision repair: base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR). BER is a major cellular defence against endogenous non-bulky DNA lesions, allowing excision and replacement of damaged DNA bases (Lindahl and Wood 1999). This includes oxidised/reduced bases, alkylated (usually methylated) bases, deaminated bases (*e.g.*, uracil, xanthine) and base mismatches. The BER process is initiated by the removal of an altered base from DNA by DNA glycosylases, *i.e.* enzymes that cleave the base-deoxyribose glycosyl bond of the damaged nucleotide (McCullough *et al.* 1999). OGG1, MYH and NTH1 are three human DNA glycosylases that are involved in the repair of oxidative damage to DNA (Wood *et al.* 2001). OGG1 glycosylase removes 8-OHdG residues opposite dC in DNA (Rosenquist *et al.* 1997). MYH glycosylase has an anti-mutagenic role by removing misincorporated dA opposite 8-OHdG in the daughter strand during replication. NTH1 glycosylase removes oxidized pyrimidines, such as thymine glycol and cytosine glycol (Lindahl and Wood 1999). The resulting base-free site (or AP) site is removed by an AP endonuclease 1 (APE1). The resulting 3'-hydroxyl group can then be extended by a DNA polymerase (β or δ) and sealed by a DNA ligase (I or III) (reviewed by Lindahl 2000).

In cases where the base damage is removed by a glycosylase/AP lyase that cleaves the phosphodiester bond 3' to the AP site, APE1 endonuclease cleaves the 5' bond to the site and recruits DNA Polymerase β, which fills in the one-nucleotide gap. This gap is subsequently ligated by the DNA ligase I/XRCC1 complex. This pathway is usually referred to as the short-patch pathway. When the AP site is generated by hydrolytic glycosylases or by spontaneous hydrolysis, repair usually proceeds through an alternative pathway, the long-patch pathway. APE1 cleaves the phosphodiester bond 5' to the AP site and then a combination of RFC, PCNA and Polδ/ϵ carries out repair synthesis. This

involves displacing the strand 3' to the nick to produce a flap of 2–10 nucleotides, which is cleaved off by FEN1 endonuclease. The patch is ligated by DNA ligase I (reviewed by Sancar *et al.* 2004).

It is worth noting that the enzymes responsible for the later stages of BER (*i.e.* DNA polymerisation and ligation) are also involved in the repair of single-strand breaks in the DNA, which has some additional steps like the temporary protection of the DNA single-stranded interruptions by the poly(ADP-ribose) polymerase 1 (PARP1) (Lindahl and Wood 1999).

NER is the major repair system responsible for the removal of helix-distorting lesions or bulky adducts, such as the pyrimidine dimers induced by UV radiation, adducts resulting from polycyclic aromatic hydrocarbons and DNA crosslinks produced from chemotherapeutic agents like cisplatin. There are two forms of NER, global genome repair (GGR) and transcription-coupled repair (TCR). Actively transcribed regions of DNA are repaired much faster than the non-transcribed regions by TCR. TCR acts on lesions that block RNA polymerase II during transcription by recruiting the appropriate repair proteins to the lesion and removing the stalled RNA polymerase. On the other hand, GGR exerts its function throughout the entire genome, regardless of whether the genes are active or inactive. As reviewed by Lindahl and Wood (1999) and Sancar *et al.* (2004), both NER mechanisms involve four steps: a) the recognition of DNA damage and recruitment of a multiprotein complex around the lesion; b) dual incisions bracketing the lesion by specific nucleases, causing the release of an oligonucleotide containing the damage (24-32 nucleotides in eukaryotes); c) repair synthesis to fill in the resulting gap; and d) ligation. Mutations in some of the genes involved in NER are the cause of several rare genetic diseases. For example, Cockayne Syndrome patients are defective in TCR, but proficient in GGR. Individuals with the rare hereditary disorder *xeroderma pigmentosum* are defective in NER and have a much higher probability of developing skin cancer (De Boer and Hoeijmakers 2000).

Mismatch repair (MMR) eliminates base-base mismatches or insertion/deletion loops that arise during DNA synthesis as a consequence of base misincorporation or strand slippage by DNA polymerase, respectively. MMR proteins detect the error on the newly

synthesised strand and replace it with the correct sequence. Mutations in the human MMR genes are correlated with an increased susceptibility to colon cancer (Peltomaki 2001a, b).

1.2.4. Adaptive response

The adaptive response may be defined as an ability of cells to better resist high "challenging" doses of a damaging agent when first exposed to a lower, non-toxic "conditioning" dose of that particular agent. Current evidence suggests that DNA damage resulting from the low-dose pre-exposure generates an unknown signal that is able to induce the adaptive response. This consists in the activation of signalling pathways and leads to *de novo* protein synthesis. The result is a temporary improvement of DNA repair, presumably due to the activation of DNA repair enzymes (reviewed by Stecca and Gerber 1998). Cellular adaptation may also result from the transcriptional activation of other stress-response genes, such as APE1 (Grosch *et al.* 1998), haem oxygenase 1 (HO-1) (Vile *et al.* 1994) or ferritin (Balla *et al.* 1992). Mammalian cell lines can cross-adapt to a variety of DNA damaging agents, such as radiation (Olivieri *et al.* 1984), oxidative stress (Laval 1988), alkylating compounds (Samson and Schwartz 1980) or heat (Henle *et al.* 1978).

1.3. Iron homeostasis

1.3.1. Iron metabolism in humans

Iron is a component of several metalloproteins involved in crucial metabolic processes in living cells and organisms, including oxygen sensing and transport (haemoglobin and myoglobin), energy metabolism (cytochromes, mitochondrial aconitase, Fe-S proteins of the electron transfer chain) and DNA synthesis (ribonucleotide reductase). As a result, iron is essential for the survival and proliferation of all eukaryotic cells. The biological importance of iron is related to its properties as a transition metal, namely its ability to readily undergo one-electron redox reactions between the ferric (Fe^{3+}) and ferrous (Fe^{2+}) states. However, these same properties explain its potential toxicity, as in aerobic conditions iron can catalyse the formation of damaging radical species if not appropriately sequestered (Richardson and Ponka 1997; Papanikolaou and Pantopoulos 2005).

Despite being an essential requirement for living organisms, iron bioavailability is limited due to the fact that, under aerobic conditions, ferrous iron is readily oxidised in

solution to ferric iron, which in turn is insoluble at physiological pH and precipitates into ferric hydroxide polymers. To overcome this problem, cells have developed iron complexing agents that serve to solubilise iron in the surrounding media, deliver it to the cells and transport it. In this respect, bacteria synthesize different iron chelating agents, siderochromes, some of which have extremely high affinity for iron. In plants iron is transported complexed with low molecular weight chelators, such as citrate and malate. On the other hand, vertebrates synthesise iron chelators in the form of proteins. A family of proteins with very high binding affinity for iron occurs in extracellular fluids, including: transferrin, the major iron-binding protein in the plasma; lactoferrin, found in several body fluids and in milk and produced by phagocytic cells; ovotransferrin (or conalbumin), the iron-binding protein of avian egg white; and melanotransferrin. In normal conditions, extracellular iron circulates in the plasma bound to transferrin. Transferrin has a very high affinity for ferric iron and transferrin-bound iron is thought to be kept in a non-reactive state. On the other hand, cells store the iron that is not required for immediate use in a protein with lower affinity but higher storage capacity, ferritin (Morgan 1981; Hentze *et al.* 2004).

The human body contains approximately 3-5 g of iron (55 and 45 mg per kilogram of body weight in adult men and women, respectively). About two-thirds of the body iron (~2.5 g) are present as haem (a molecule composed of protoporphyrin IX and ferrous iron) in haemoglobin within circulating erythrocytes, whereas the remaining is found in myoglobin, cytochromes and iron-containing enzymes (10 %) or stored in hepatocytes and reticuloendothelial macrophages, mainly within ferritin or its degradation product, haemosiderin (20-30 %). Iron bound to transferrin, in turn, corresponds to less than 0.1 % of total body iron (~3 mg). However, it is a very dynamic iron pool, with a high turnover. In humans, most of the transferrin-bound iron turnover is related with the process of erythropoiesis (25-30 mg/day). Transferrin-bound iron is transported to the bone marrow for haemoglobin synthesis in developing erythroid cells (Ponka and Lok 1999; Hentze *et al.* 2004; Papanikolaou and Pantopoulos 2005). Erythrocytes circulate in the blood for about 120 days until they become senescent. At this stage, they are engulfed by splenic macrophages and their components undergo extensive breakdown. In this process, the haem moiety is separated from haemoglobin. Whilst globin is degraded to amino acids, haem

degradation is catalysed by the microsomal enzyme haem oxygenase. The reaction generates iron, carbon monoxide and biliverdin IXα, which is further converted to the antioxidant bilirubin IXα (Sassa 2004; Ryter *et al.* 2006). The iron liberated during this process is exported back to plasma transferrin. Despite the daily requirement of 25-30 mg iron for erythropoiesis and other uses, the daily dietary iron uptake corresponds to no more than 1-2 mg, which can only compensate for iron loss through cell desquamation. Indeed, the human body has no special mechanisms for iron excretion and most of the iron loss occurs through cell desquamation from the skin, the gastrointestinal and the urinary tracts. So, the vast majority of the available transferrin-bound iron pool is accounted for by macrophage recycling (Morgan 1981; Ponka and Lok 1999; Hentze *et al.* 2004; Papanikolaou and Pantopoulos 2005).

1.3.2. Iron transport and uptake mechanisms

i) Dietary iron uptake

Dietary iron uptake occurs in the apical surface of duodenal absorptive cells (enterocytes). Non-haem food iron is present mainly in the Fe^{3+} form, which is insoluble and needs to be reduced to Fe^{2+} for transport across the intestinal epithelium. This reduction is operated by an ascorbate-dependent duodenal ferrireductase, Dcytb, which belongs to the cytochrome b561 family of proteins. Subsequently, Fe^{2+} is transported through the apical membrane by the divalent metal transporter 1 (DMT1; also known as Nramp2) (McKie *et al.* 2002). Once inside the enterocytes, iron is then transported across the basolateral membrane by ferroportin 1 (or IREG1) and delivered to plasma transferrin.

ii) Transferrin-dependent iron uptake

In vertebrates, iron is transported between sites of absorption, storage and utilisation by a monomeric glycoprotein with a molecular mass (M_r) of 80 kDa, transferrin. Each *apo*-transferrin molecule can bind up to two atoms of ferric iron with very high affinity (K_d = 10^{-23} M). Nevertheless, binding is reversible and pH-dependent. Whilst binding is maximal above pH 7.0, iron starts to dissociate from the molecule at about pH 6.5 and is completely disassociated by pH 4.5 (Morgan 1981). The only known functions of transferrin are related to its affinity for iron and involve iron transport in the plasma and interstitial fluid and iron exchange between cells and the extracellular fluid. Indeed, transferrin circulates in the

plasma and interstitial fluid, accepting iron absorbed from the diet, from storage sites or released from degraded iron-containing molecules (the major source being the degradation of senescent erythrocytes in the cells of the reticuloendothelial system, as discussed in section 1.3.1) and donating it to proliferating cells, especially immature erythrocytes, and to iron-storage cells (Morgan 1981).

Cellular iron uptake involves the binding of transferrin to its receptor at the cell surface. The transferrin receptor (TFRC) is expressed in nearly all cell types, with the exception of mature erythrocytes and some other terminally differentiated cells. High levels of the receptor are found in immature erythroid cells, placental tissues and rapidly dividing cells (reviewed by Ponka and Lok 1999). The human TFRC gene is located on chromosome 3, has a size of about 32 kb and contains 19 exons. The TFRC protein is encoded by a 4.9 kb mRNA (Kühn *et al.* 1984). This includes a large (~2,600 bases) untranslated region (UTR) at the 3' end of the mRNA (Kühn *et al.* 1984) that is required for the iron-dependent regulation of receptor expression (Owen and Kühn 1987) (further described in Section 1.3.6). In humans, the TFRC is a homodimer of two identical transmembrane subunits of approximately 95 kDa (Kühn *et al.* 1984). Each subunit is divided into three domains, a large C-terminal extracellular domain of 671 amino acids, a small intra-membrane domain of 28 amino acids and a cytoplasmic N-terminus of 61 amino acids. The two subunits are joined by two disulfide bonds (Cys-89 and Cys-98) in the central domain. Each receptor subunit binds one molecule of transferrin at its extracellular carboxy-terminal domain. This domain contains several glycosylation sites, which are required for the binding activity of the receptor (reviewed by Ponka and Lok 1999).

In humans, the main site of transferrin synthesis is the liver. Transferrin is distributed throughout most of the extracellular fluid of the body, including plasma, the interstitial fluid, lymph, pleural, cerebrospinal, ascitic and oedema fluid. The mean transferrin content of normal adult serum is approximately 2.3 mg/ml (Morgan 1981). On the other hand, analyses of rat mesenteric tissue showed that transferrin concentrations in the interstitial fluid are approximately one third lower than those in the plasma (Barber *et al.* 1990). Plasma transferrin concentrations are elevated during childhood and pregnancy, in iron-deficient anaemia and as a consequence of oestrogen administration. Conversely, plasma transferrin levels decline as a result of iron overload and liver malfunction, protein

malnutrition and other disorders (Morgan 1981). Normal plasma transferrin concentration is about 50 μM, of which the predominant species is *apo*-transferrin (50 %). The major iron species is monoferric transferrin (40 %) and only approximately 10 % of the total transferrin population corresponds to diferric transferrin (*i.e.* 5 μM) (reviewed by Ponka and Lok 1999). However, the binding affinity of transferrin to its receptor is related to the iron content of the protein, with diferric transferrin having the highest affinity, monoferric transferrin having intermediate affinity and *apo*-transferrin the lowest (Young *et al.* 1984). For this reason, the diferric transferrin concentration in normal plasma is enough to saturate all TFRCs and consequently iron is delivered to cells mainly by diferric transferrin, while monoferric or *apo*-transferrin are not able to compete significantly for the receptor (reviewed by Ponka and Lok 1999).

The mechanism of cellular iron uptake in vertebrates is well-studied and involves binding of transferrin to the TFRC at the cell surface, internalisation of the transferrin-TFRC complex by receptor-mediated endocytosis and release of iron from transferrin at acidic pH (pH ~ 5.5) within the endocytic vesicle (Figure 1.4). Once released from transferrin, iron is transported through the endosomal membrane and becomes part of a cytosolic labile pool, which is available for storage in ferritin or for the synthesis of iron-containing proteins. Meanwhile, the iron free transferrin-TFRC complex recycles back to the cell surface, where *apo*-transferrin is released from the cell and becomes available for an additional cycle of iron binding and endocytosis (reviewed by Klausner *et al.* 1983; Ponka and Lok 1999). Recent studies have helped clarify the mechanism of the release of iron within the endosomes. Acidification of endosomes by a proton pump induces conformational changes in the transferrin-TFRC complex and causes iron release. The ferric iron released from transferrin is subsequently reduced to ferrous iron by a ferrireductase and transferred to the cytoplasm through a divalent transporter at the endosomal membrane, DMT1 (reviewed by Ponka and Lok 1999; Hentze *et al.* 2004). An endosomal ferrireductase (Steap 3) was identified in erythroid cells (Ohgami *et al.* 2005).

Small amounts of TFRCs are found in the serum and their concentration seems to be proportional to the estimated total body mass of TFRCs (Cook *et al.* 1993; Ahluwalia 1998). This TFRC in circulation corresponds to a soluble truncated form of the receptor of approximately 85 kDa, lacking the cytoplasmic and transmembrane domains (Shih *et al.*

1990). Shedding of the extracellular fragment is mediated by a membrane metalloprotease (Kaup *et al.* 2002). As expected, serum TFRC levels are elevated in patients with iron deficiency anaemia (Ferguson *et al.* 1992).

iii) Non-transferrin bound iron uptake

Under normal conditions, the total iron-binding capacity of the transferrin in plasma and interstitial fluid is only about 30 % saturated with iron, so it is believed that transferrin is capable of binding all extracellular iron (Morgan 1981). However, in pathological conditions associated with iron overload, iron gradually saturates the binding capacity of transferrin and forms redox-active complexes with low molecular weight chelators. Indeed, micromolar concentrations of non-transferrin-bound iron (NTBI) are commonly found in plasma during acute iron poisoning and in severe idiopathic haemochromatosis, thalassaemia, Bantu siderosis and congenital atransferrinemia (Hershko and Peto 1987).

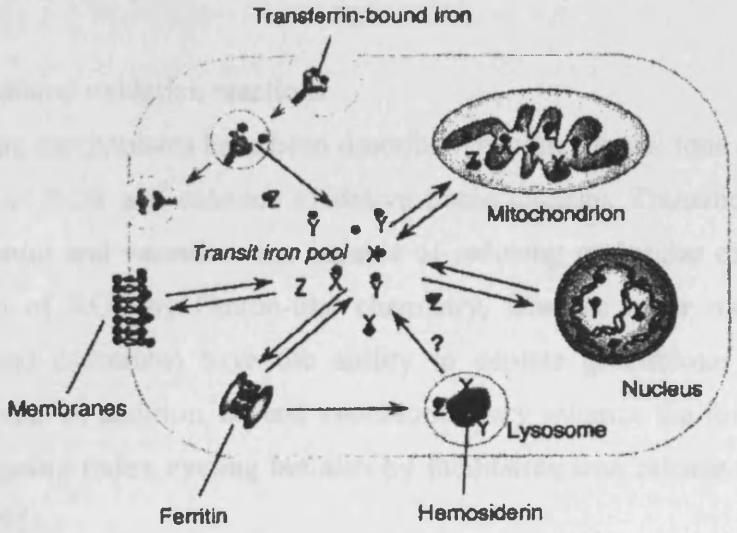


Figure 1.4. Overview of cellular iron metabolism (Petrat *et al.* 2002). Iron enters cells bound to transferrin by receptor-mediated endocytosis. Iron release from transferrin occurs within the endocytic vesicle. Once released from endosomes, iron becomes loosely bound to low molecular weight chelators (X, Y, Z). This pool of labile iron is associated with cellular membranes, with cellular organelles such as the mitochondria, with DNA in the nucleus, or in transit to the storage protein ferritin or to the synthesis of functional iron-containing proteins.

NTBI is thought to consist of a heterogeneous population of low-molecular weight iron species, predominantly in the Fe^{3+} state, that may be non-specifically bound to serum proteins (*i.e.* not bound to transferrin, ferritin or haemoglobin) in patients with saturated transferrin (Graham *et al.* 1979; Hershko and Peto 1987). NTBI in the serum or plasma of individuals with idiopathic haemochromatosis exists mainly as a complex with citrate (Grootveld *et al.* 1989).

Whilst receptor-mediated endocytosis of transferrin is thought to be the primary route of iron transport in normal conditions, NTBI can enter cells by poorly characterised, non-specific membrane transporters (Richardson and Baker 1992; Oshiro *et al.* 1993; Papanikolaou and Pantopoulos 2005). In fact, several cell types are able to take up iron from iron-complexes in culture medium in the absence of serum or transferrin (Richardson and Ponka 1997). Uptake of NTBI is a low-affinity, saturable and temperature-dependent process. It seems to involve binding of a soluble ferric iron chelate to the cell-surface and its reduction to ferrous iron by a membrane ferric reductase, followed by uptake of the reduced form across the plasma membrane (Jordan and Kaplan 1994).

1.3.3. Iron-mediated oxidation reactions

Different mechanisms have been described by which metal ions are able to facilitate the formation of ROS and enhance oxidative tissue damage. Transition metals like iron, copper, chromium and vanadium are capable of reducing molecular oxygen and facilitate the production of ROS by Fenton-like chemistry, whereas other metal ions (mercury, nickel, lead and cadmium) have the ability to deplete glutathione and protein-bound sulfhydryl groups. In addition, several xenobiotics may enhance the formation of ROS not only by undergoing redox cycling but also by facilitating iron release (reviewed by Stohs and Bagchi 1995).

The role of iron in the deleterious oxidation of biomolecules has been reviewed in the recent years (*e.g.* Welch *et al.* 2002). The toxicity of iron (and other transition metals) is a consequence of its ability to switch readily between two stable oxidation states, ferric (Fe^{3+}) and ferrous (Fe^{2+}), and can be largely explained by its participation in Fenton- and Haber-Weiss-type reactions. Fenton first reported the oxidising properties of a mixture of iron (ferrous salt) and H_2O_2 more than a century ago (Fenton 1894). In this reaction, a

transition metal ion reacts with H_2O_2 to yield hydroxyl radical, the most potent oxidising species in biological systems, and the oxidised metal ion (equation 4). Subsequently, Haber and Weiss reported that free radical intermediates are involved in the Fenton reaction (Haber and Weiss 1934). In these reactions, superoxide anion converts the oxidised metal ion back to its reduced form (equation 10) and this reacts with H_2O_2 to produce hydroxyl radicals (equation 11). The whole process can be summarised as follows, where iron is the transition metal catalyst:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO^{\bullet} \qquad [4]$$

$$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2 \qquad [10]$$

(Net reaction)

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe} O_2 + HO^{\bullet} + OH^- \qquad [11]$$

With the exception of exposure to ionising radiation, most of the hydroxyl radical produced *in vivo* is thought to originate from the metal-dependent decomposition of H_2O_2 (Halliwell and Gutteridge 1990). Haber-Weiss reactions can be particularly important during phagocytosis, when large amounts of superoxide anion are produced in a respiratory burst (Forman and Thomas 1986).

ROS-mediated, iron-dependent injury processes include DNA damage, lipid peroxidation and protein modification. The free radicals produced in iron-mediated processes can modify DNA by causing single- and double-strand breaks, depurination/depyrimidation or chemical modification of the bases or sugar (reviewed by Welch *et al.* 2002). Iron-mediated oxidation of DNA is a site-specific process that involves the binding of iron or an iron chelate to DNA, either at the phosphate backbone or at a base, where iron will catalyse the repetitive formation of the hydroxyl radical resulting in modification of the DNA structure (Floyd 1981). Reducing agents like superoxide anion, ascorbate or GSH could reduce the iron catalyst, which in turn would react with H_2O_2 and yield the hydroxyl radical by Fenton reaction. Hydroxyl radicals are very reactive and short-lived, so they would attack the DNA molecule at the vicinity of the site of formation by causing strand breaks, by abstracting a hydrogen atom or by adding a double bond. This mechanism would lead to DNA damage being formed preferentially at specific sites, namely where the metal is bound. Likewise, as the metal catalyst can undergo repeated

cycles of reduction and oxidation in the presence of reducing agents, a 'multi-hit' mechanism can be envisaged that would take place close to the metal binding site. This multi-hit mechanism would account for a higher incidence of double-strand breaks than what would be expected in case of random-hits and consequently lead to loss of genetic information (Chevion 1988).

The bleomycin (BLM)-induced DNA damage is a well-studied mechanism of site-specific metal-dependent damage to DNA. BLM is a glycopeptide antibiotic produced by *Streptomyces verticillus* that is able to chelate transition metal ions and has been used clinically against several human cancers. Its clinical action is thought to be due to binding of BLM to DNA, where it affects helix unwinding, replication and transcription, and may cause single- and double-strand breaks. The ability of BLM to damage DNA is dependent on the presence of certain transition metal ions (like iron) within the DNA- BLM complex. Indeed, the species that attacks DNA is thought to be a ferric peroxide BLM-$Fe^{3+}O_2H^-$, which decomposes to release hydroxyl radical (reviewed by Claussen and Long 1999).

A BLM-based assay has been employed to measure the presence of catalytic iron in biological fluids. BLM on its own cannot mobilise iron from metalloproteins such as haemoglobin, transferrin or ferritin at physiological pH. So BLM-detectable iron in body fluids is believed to correspond to iron bound to low molecular mass chelators or loosely bound to proteins and which is available for stimulating free radical formation (Evans and Halliwell 1994). Importantly, the BLM assay does not measure iron bound to transferrin or ferritin and therefore blood serum or plasma from healthy human individuals does not contain BLM-detectable iron (reviewed by Halliwell and Gutteridge 1990).

Mechanisms of site-specific metal-induced damage have also been described in proteins; again, a metal (usually copper or iron) bound to the protein serves as the centre for hydroxyl radical formation. This was shown to be a cause of enzyme inactivation (reviewed by Chevion 1988; Welch *et al.* 2002).

In the recent years, it has been hypothesised that certain parts of the genome may be more vulnerable to Fenton reactions (Toyokuni 2002). Tanaka *et al.* (1999) reported the existence of chromosomal areas with elevated frequency of allelic loss and identified the tumour suppressor genes p16 and p15 as two major targets in a ferric nitrilotriacetate-induced rat renal carcinogenesis model.

Whilst a number of different metal species can catalyse Fenton-type reactions in acute metal poisoning, only Fe^{2+}-mediated hydroxyl radical formation seems to be physiologically relevant (Halliwell and Gutteridge 1990; Barbouti *et al.* 2001). Copper, for example, is more soluble than iron in physiological conditions and it is a better catalyst of Fenton reactions (Chevion 1988). However, copper is not so ubiquitous in the human body as iron and eukaryotic cells are thought to chelate intracellular copper efficiently (Rae *et al.* 1999), so 'free' copper may not be available for participating in free radical formation. Naturally, for iron to facilitate the formation of ROS via the Fenton reaction it must be present in a 'free' or catalytically active form. For iron to be 'free' it is necessary that at least one coordination site is open or occupied by a readily dissociable ligand. In the human body, however, iron is safely bound to proteins which tightly complex all six coordination sites of iron: transferrin in the plasma, ferritin in cells, haemoglobin in red blood cells and myoglobin in muscles. For example, serum from healthy individuals contains no bleomycin-detectable iron (*e.g.* Gutteridge *et al.* 1985). Therefore, some authors have proposed that the effects of Fenton-reactions *in vivo* are limited by the reduced availability of iron to stimulate hydroxyl radical formation. Nevertheless, transferrin is often completely saturated in thalassemic patients (Hershko and Peto 1987) and individuals with idiopathic haemochromatosis, leading to the appearance of bleomycin-detectable, NTBI in the serum of these individuals (*e.g.* Gutteridge *et al.* 1985). Importantly, the BLM-detectable NTBI found in individuals with iron overload stimulates lipid peroxidation of phospholipid liposomes in the presence of AA and formation of hydroxyl radicals (Gutteridge *et al.* 1985), which strongly suggests that NTBI is implicated in the toxic effects of iron overload. Iron bound to citrate, ATP or ADP remains 'free' as these ligands cannot complex all of its coordination sites (Graf *et al.* 1984). A small pool of such labile iron exists in cells and is available to catalyse deleterious free radical reactions (see Section 1.3.5). In addition, it is possible that oxidative stress may increase iron availability by superoxide-mediated iron mobilisation from ferritin (see Section 1.3.4) or by iron release during H_2O_2-mediated degradation of haem-containing proteins. Moreover, tissue injury can exacerbate free radical reactions by liberating catalytic metal ions from damaged cells into the surrounding environment (discussed by Halliwell and Gutteridge 1990). In all

cases, the presence of physiologically relevant reducing agents could increase iron availability even further by recycling Fe^{2+}.

Iron toxicity is involved in several human pathologies, such as acute iron poisoning, ischemia-reperfusion injury to different organs, idiopathic haemochromatosis, congenital atransferrinemia, as well as secondary iron overload in β-thalassemia. Hereditary haemochromatosis (HH) is an autosomal recessive disorder that is characterised by excessive absorption of dietary iron, leading to increased iron deposition in the liver and in several other body organs and tissues (pancreas, heart, joints and pituitary gland). HH patients often develop arthritis, hypogonadism, diabetes, liver fibrosis, cirrhosis, primary hepatocellular carcinoma and cardiac failure. Treatment can be achieved with phlebotomy, providing that there is an early diagnosis and an adequate erythropoietic reserve. Most cases of HH are attributed to mutations in the haemochromatosis gene (HFE), although mutations in the TFRC2 gene have also been implicated. Importantly, approximately one-third (35 %) of all Europeans and Americans are thought to carry at least one mutant allele for the HFE gene and the estimated prevalence of HH is about 50 per 10,000 persons (Hanson *et al.* 2001). Juvenile haemochromatosis presents a more severe, early-onset phenotype and is associated with mutations in the gene encoding hepcidin. Thalassaemia is a group of genetic disorders where haemoglobin synthesis is impaired, the most common of which is thalassemia major. Individuals with ineffective erythropoiesis have a compensatory absorption of iron from the gastrointestinal tract, which develops into iron overload pathology. In addition, treatment is achieved through frequent blood transfusion, which contributes more iron to the organism through the haemoglobin contained in transfused red blood cells. Consequently, patients develop secondary iron overload, with excessive iron deposition in tissues. Patients suffer from growth failure, hypogonadism and bone and heart disease. Secondary iron overload is treated with iron chelation therapy, most commonly by intravenous administration of desferrioxamine (DFO), which is essential to remove the iron deposits (Papanikolaou and Pantopoulos 2005).

In addition, metals, iron in particular, are thought to be involved in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and prion disease. Indeed, high oxygen consumption, high levels of iron and ascorbate, low levels of antioxidants and a tendency to accumulate metals make the brain particularly prone to

oxidative damage (Gaeta and Hider 2005). Iron is also thought to promote carcinogenesis (Huang 2003). Finally, metal-induced oxidative stress causes the activation of cellular signalling pathways and nuclear transcription factors, eventually leading to cell cycle arrest and/or apoptosis (Leonard *et al.* 2004).

1.3.4. Iron chelation

Iron-mediated damage mechanisms can be circumvented by the use of a chelating agent such as DFO or diethylenetriaminepentaacetate (DTPA). These chelators can prevent damage by removing the metal from the biological binding site and/or by keeping the metal in the oxidised state. DFO is a clinically used ferric iron chelator obtained from *Streptomyces pilosus* (Keberle 1964; Halliwell 1989). In the last decades it has been the most widely used chelator in the treatment of acute iron poisoning and of β–thalassaemia and other iron overload disorders (Chaston and Richardson 2003). The drug is relatively safe and, when administered by continuous subcutaneous infusion (therapeutically relevant doses are 20-100 μM), it leads to efficient iron excretion in the urine (Keberle 1964). DFO forms a very stable complex with Fe^{3+}, thereby keeping iron ions in the oxidized state and not allowing them to catalyse redox reactions (Halliwell 1989). DFO permeates into cells very slowly (Halliwell 1989), scavenges some of the intracellular labile iron (*e.g.* Epsztejn *et al.* 1997) and protects cells from H_2O_2-induced cytotoxicity (Starke and Farber 1985). DFO can induce cell cycle arrest (*e.g.* Doulias *et al.* 2003) and, in some cell types, apoptosis (Fukuchi *et al.* 1994).

DTPA is an aminocarboxylate that has been used clinically used as a good chelator of Fe^{3+}. As for DFO, intravenous or intramuscular administration of DTPA leads to efficient iron excretion in the urine of patients with iron overload (Fahey *et al.* 1961). However, DTPA has poor selectivity for iron and patients often develop zinc deficiency as a side effect. Due to its hydrophilic nature, DTPA remains in the extracellular space (discussed in Porter 1989). As discussed by Halliwell and Gutteridge (1986), DTPA is not a general inhibitor of iron-mediated radical reactions, but an inhibitor of superoxide-driven Fenton reactions. Thus, whilst Fe^{2+}-DTPA chelates are known to catalyse hydroxyl radical formation from H_2O_2 (Cohen and Sinet 1982), DTPA blocks reduction of Fe^{3+} by superoxide, thereby reducing superoxide-driven Fenton chemistry (Butler and Halliwell

1982). Nevertheless, other reductants more powerful than superoxide reduce the Fe^{3+} complex and thus cause hydroxyl radical formation in the presence of H_2O_2.

Some of the toxicity associated with iron chelators is explained by the redox cycling of iron complexes between Fe^{2+} and Fe^{3+}. In order to achieve efficient protection against radical formation, iron should be coordinated in a way to prevent access of oxygen and H_2O_2. Most hexadentate chelators, such as DFO, completely mask the surface of iron, keeping it in a safe, non-reactive state. However, some hexadentate chelators are not of sufficient size to completely protect the bound iron. In that case, the iron-chelate complex may even increase the ability of iron to generate free radicals. This may happen because, whilst Fe^{3+} is only partially soluble at physiological pH, its solubility increases when complexed with the chelator. Bidentate and tridentate ligands may also increase iron availability for free radical generation by forming partially coordinated iron complexes (discussed by Gaeta and Hider 2005). Ethylenediaminetetraacetic acid (EDTA), for example, can exacerbate iron-mediated free radical production (Chaston and Richardson 2003). Although EDTA is an effective chelator of copper ions, iron-EDTA chelates have been shown to enhance hydroxyl radical formation from H_2O_2 and superoxide or ascorbate *in vitro* because the chelator increases the solubility of iron salts at physiological pH, thereby increasing the effective concentration of Fe^{3+} in solution (reviewed by Halliwell and Gutteridge 1990). So, the ability of an iron chelate to catalyse hydroxyl radical generation requires the existence of a coordination site that is free or occupied by an easily displaceable ligand such as water (Graf *et al.* 1984).

A number of compounds have been known to react as bidentate ligands with several metal ions such as ferrous, cuprous or cobaltous, yielding a stable, coloured species. Examples of these compounds are 1,10-phenanthroline, 2,2'-bipyridine and 2,6-bis(2-pyridil)-pyridine. The disodium salt of 3-(2-pyridil)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine or ferrozine (FZN) reacts with ferrous iron to form a stable magenta species that is soluble in water and has therefore been used for the quantitative determination of iron in solution (Stookey 1970). For example, FZN has been used for the determination of submicrograms of iron in human serum (Carter 1971).

1.3.5. Iron storage within ferritin

As mentioned above, iron is required for normal cell growth and proliferation. However, excess iron is potentially harmful, as it can promote the formation of free radicals via Fenton reactions. Ferritin is a ubiquitous iron-binding protein and constitutes a key defence against iron-mediated pro-oxidant reactions. Its main function is to sequester iron in a non-toxic form, consequently limiting the intracellular labile iron pool. In vertebrates, ferritin consists of two subunits, named H and L, which are encoded by separate genes. The empty ferritin molecule (*apo*-ferritin) consists of a protein shell containing 24 subunits of molecular weight 18,500 Da each and six transprotein channels leading to an inner core. Iron is transported through these channels and stored in the central core of the molecule as hydrous ferric oxide. Each *apo*-ferritin molecule has affinity for up to 4500 iron atoms (Torti and Torti 2002). It is believed that ferritin stores iron in a relatively inert form until it is needed for iron-dependent processes such as the synthesis of haem proteins. Thus, iron is thought to be safely stored in the 3+ oxidation state. However, ferritin contains small amounts of a dissociable form of iron, which is in the ferrous state. The ferrous iron content increases when ferritin is incubated with biological reducing agents such as GSH, ascorbate or cysteine. *In vitro* experiments have shown that ferrous iron can be mobilised from ferritin at physiological pH (Mazur *et al.* 1955; Dognin and Crichton 1975). Ferritin iron can be released either by direct chelation, but this is, however, a very slow process. Alternatively, iron can be released by reduction in the presence of a Fe^{2+} chelating agent (equations 12 and 13) (Funk *et al.* 1985).

$$\text{Ferritin:}Fe^{3+}_{(n)} + \text{reductant} \longrightarrow \text{ferritin:}Fe^{3+}_{(n-1)}Fe^{2+} \quad [12]$$

$$\text{Ferritin:}Fe^{3+}_{(n-1)}Fe^{2+} + \text{chelator} \longrightarrow \text{ferritin:}Fe^{3+}_{(n-1)} + Fe^{2+}\text{chelate} \quad [13]$$

For iron to be mobilised from ferritin, a relatively long-lived reducing agent has to penetrate the ferritin channels, reduce Fe^{3+} in the ferritin core to Fe^{2+} and subsequently release it from the protein. The reduction potential of ferritin iron at pH 7.0 is -190 mV (Watt *et al.* 1985), so iron can be mobilised by species with a reduction potential more negative than this. It seems clear that for an agent to cause iron release from ferritin, it must either have an appropriate reducing potential or be an iron chelator. The identities of relevant endogenous physiological reductants and low molecular weight chelators have not been established yet. Nevertheless, several studies have shown that the superoxide anion

can mobilise iron from ferritin and promote oxidative damage. As superoxide is thought to be produced under situations that are known to mobilise iron from ferritin *in vitro* (oxidase activity, radiolysis, metal-catalised oxidations or redox cycling xenobiotics) and as it is known to occur *in vivo*, it was suggested that it may be a relevant reductant (Reif 1992). Alternatively, ascorbate could replace superoxide. Indeed, test tube experiments have also shown that ascorbate, but not dehydroascorbate or H_2O_2, mobilised iron from ferritin in the presence of oxygen (Bienfait and van den Briel 1980). It was proposed that ascorbate is able to diffuse into ferritin, reduce ferric to ferrous iron and chelate it, assisting in the diffusion of the ferrous species out of ferritin (Tufano *et al.* 1981). Alternatively, superoxide anion generated during the aerobic oxidation of ascorbate could reduce ferritin iron. In fact, in at least one experimental system ascorbate-mediated iron release from ferritin was markedly inhibited by SOD (Boyer and McCleary 1987).

1.3.6. Intracellular labile iron pool

As mentioned before, iron is simultaneously essential for life and potentially cytotoxic. For this reason, the majority of the intracellular iron is safely bound to storage, functional or transport proteins. However, a small pool of cellular iron is bound to physiologically relevant low molecular weight organic chelators with a low affinity for iron ions (*e.g.* organic acids such as citrate or phosphate esters such as ATP, ADP or GTP) or loosely associated with macromolecules (*e.g.* the polar head groups of membrane lipids or DNA) (reviewed by Halliwell and Gutteridge 1990). This pool was originally named 'labile iron pool' (LIP) (Greenberg and Wintrobe 1946) but other terms have since then been employed, including 'free iron', 'redox-active iron', 'low molecular weight iron' or 'transit pool'. More recently the LIP has also been termed 'chelatable iron' because most methods of detection rely on the use of chelating molecules. In fact, the existence in many different cell types of low molecular weight iron complexes that are available for the synthesis of iron-containing proteins involved in different processes (*e.g.* DNA synthesis, haemoglobin synthesis, tyrosine and proline hydroxylase) or for uptake by ferritin or chelators has been known for a few decades (Jacobs 1977), but the exact nature of this iron pool still requires clarification. It is thought to originate from iron released from endosomes after the receptor-mediated endocytosis of transferrin-bound iron (described in Section 1.3.2) that is

in transit to the storage protein ferritin or to the synthesis of functional iron-containing proteins. In human erythroleukemia K562 cells, the transit time of iron taken up from transferrin through the chelatable pool was estimated to be approximately 2 hours (Breuer *et al.* 1995). The LIP is believed to occur mainly in the reduced form (Fe^{2+}) under physiological conditions (Breuer *et al.* 1995) and, as illustrated in Figure 1.4, it is associated with the cytosol, with cellular organelles such as the mitochondria, and with DNA in the nucleus (Petrat *et al.* 2002). This transit pool of chelatable iron is thought to catalyse the generation of highly reactive oxygen species within mammalian cells, namely the formation of the hydroxyl radical from H_2O_2. In fact, iron complexed with organic chelators such as ATP, histidine, EDTA or citrate is able to catalyse the formation of ROS (reviewed by Stohs and Bagchi 1995).

1.3.7. Iron-regulated gene expression

As mentioned above, cellular iron is at the same time essential for life and potentially cytotoxic. For this reason, iron transport and storage are two tightly regulated processes. Even though transferrin (a protein with high affinity) and ferritin (a protein with high capacity) keep iron safely bound while transported and stored, respectively, a pool of redox-active iron in transit is known to exist within the cells (see Section 1.3.6). Whilst a reduction in the intracellular LIP could lead to iron deficiency, the expansion of the LIP could have significant deleterious consequences. Therefore, mammalian cells regulate iron levels tightly by adjusting the expression of iron uptake and storage proteins. This, in turn, is mediated by *trans*-acting iron regulatory proteins (IRPs) that bind to RNA motifs known as iron-responsive elements (IREs).

Two IRPs occur in mammalian cells and these are referred to as IRP1 and IRP2. Both bind IREs with great affinity. IRP1 is a bifunctional protein containing a [4Fe-4S] cluster. The protein function is regulated by changes in the integrity of the iron-sulfur cluster, which in turn are determined by the intracellular iron availability. When iron levels are high, the cluster is intact and IRP1 is a cytosolic aconitase (c-acon) enzyme with no RNA binding affinity. However, in iron depletion conditions the [4Fe-4S] cluster disassembles, hence the aconitase activity is lost and the protein becomes capable of binding RNA at IREs (Klausner and Rouault 1993). In contrast, IRP2 does not have an

iron-sulphur cluster and the enhancement of its RNA binding activity by iron depletion seems to require *de novo* protein synthesis (reviewed by Templeton and Liu 2003).

IREs occur in single copies in the 5'-UTRs of the mRNAs of ferritin H and L chains, ferroportin, mitochondrial aconitase, succinate dehydrogenase and δ-aminolevulinic acid synthase. The binding of IRPs to IREs in the 5'-UTRs is enhanced by iron depletion and blocks the progression of the translational machinery, thereby inhibiting the protein synthesis from the above-mentioned genes. On the other hand, IREs are found in the 3'-UTRs of TFRC and DMT1 mRNAs, where they cannot block translation. In this case, the binding of IRPs enhanced by iron depletion protects the mRNAs from degradation by unidentified nucleases and increases the expression of these genes (reviewed by Templeton and Liu 2003; Hentze *et al.* 2004).

A consensus IRE motif was defined based on sequence and secondary structure formation analysis of ferritin and TFRC mRNAs (Figure 1.5). IREs are small nucleotide sequences that form stem-loop structures. The loop is a conserved sequence of six nucleotides, with a consensus sequence 5'-CAGUGN-3', situated on top of a stem composed of any paired bases. The stem has a variable length, but it invariably contains an unpaired cytidine situated six bases 5' to the six nucleotide loop (Klausner *et al.* 1993).

The control of the expression of iron uptake and storage proteins achieved through the interaction of IRPs with IREs in the respective mRNAs has become one of the most established mechanisms of post-transcriptional regulation (Templeton and Liu 2003). The regulation of expression of TFRC and ferritin, two crucial proteins of iron metabolism that are under the control of the IRE/IRP system, is depicted in Figure 1.5. Briefly, during iron depletion IRP loses aconitase activity and binds to IREs, causing an increase in TFRC mRNA stability and inhibiting translation of ferritin mRNA, ultimately favouring iron uptake. Conversely, in states of iron abundance IRP has aconitase activity and does not bind to IREs, causing increased ferritin mRNA translation and rapid degradation of the TFRC mRNA, *i.e.* ultimately favouring iron storage and inhibiting iron uptake (reviewed by Ponka and Lok 1999; Hentze *et al.* 2004).

The effects of iron on gene expression are not limited to the modulation of genes involved in its own homeostasis. In fact, due to the ability of the LIP to promote the formation of ROS, iron may regulate gene transcription through the effects of ROS on

signal transduction pathways. Iron overload induces transcription of several well-known antioxidant defence genes such as glutathione peroxidase, HO-1 and metallothioein (reviewed by Templeton and Liu 2003).

1.3.8. The activation of haem oxygenase during oxidative stress

The haem molecule is a crucial component of proteins involved in oxygen transport (haemoglobin, myoglobin), mitochondrial respiration (cytochromes), drug metabolism (cytochrome P450), cellular antioxidant defences (CAT and glutathione peroxidase) and signal transduction processes (nitric oxide synthases). Free haem is, however, very toxic. Indeed, haem, like other low–molecular weight iron chelates, may promote deleterious iron-dependent reactions leading to the formation of ROS and membrane lipid peroxidation, though it remains controversial whether iron in haem proteins is available for Fenton-type reactions. Therefore, free haem is efficiently kept at very low (probably submicromolar) levels within cells. Haem oxygenases are the rate-limiting enzymes in the degradation of cellular haem to form biliverdin IXα, ferrous iron and carbon monoxide. Biliverdin IXα is subsequently reduced to bilirubin IXα by a NADPH-dependent reductase. Bilirubin, in turn, has antioxidant properties (Ryter and Tyrrell 2000; Sassa 2004).

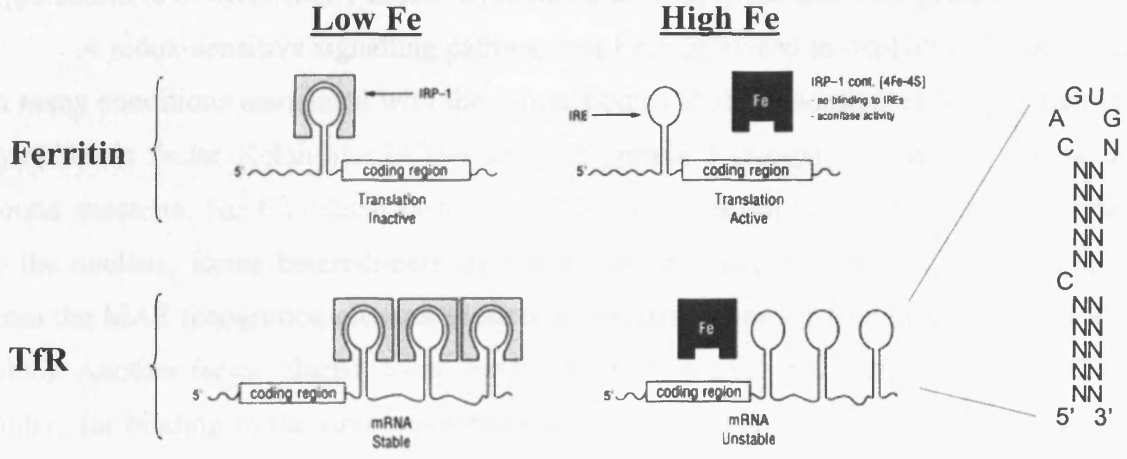


Figure 1.5. Regulation of the expression of ferritin and TFRC by intracellular iron through binding of iron regulatory proteins (IRPs) to iron-responsive elements (IREs) in the respective mRNA molecules. Adapted from Ponka and Lok (1999).

Haem oxygenases (~32 kDa) consist of two isoforms, HO-1 and HO-2, encoded by distinct genes. Whilst HO-2 is constitutively expressed in the liver, spleen, brain and testes, HO-1 is a ubiquitous stress-inducible isoform of HO that is not produced constitutively at a significant level (Ryter *et al.* 2006). The induction of HO-1 is a general response of many different organisms to stress, including hyperthermia, hypoxia, UVA radiation, heavy metals, ROS and pro-inflammatory agents (Ryter *et al.* 2006). For example, HO-1 mRNA levels are transiently increased when human fibroblasts are incubated with exogenous sources of iron (ferrous sulfate, ferric ammonium citrate or *holo*-transferrin) (Panchenko *et al.* 2000). The induction of HO-1 is thought to represent an antioxidant protective mechanism that leads to a reduction in the cellular pool of haem and haem-containing proteins, thus removing potential pro-oxidant catalysts (Vile *et al.* 1994; Rothfuss *et al.* 2001; Sassa 2004). HO-1 has therefore been used as a biomarker of oxidative stress *in vivo*. For example, the up-regulation of HO-1 by ROS has been observed in humans following endurance exercise (Niess *et al.* 1999) and hyperbaric oxygen treatment (Speit *et al.* 2000). The importance of HO-1 function was highlighted by studies of HO-1 deficiency. Growth impairment, anaemia, endothelial injury and iron deposition in renal and hepatic tissue have been observed in HO-1 targeted mice (Poss and Tonegawa 1997 a) and HO-1-deficient humans (Yachie *et al.* 1999). Furthermore, embryonic fibroblasts from these mice were hypersensitive to cytotoxicity caused by hemin and H_2O_2 (Poss and Tonegawa 1997 b).

A redox-sensitive signalling pathway has been proposed to explain HO-1 activation in many conditions associated with the production of ROS. Oxidation of SH groups in the cytoplasmic factor Kelch-like ECH-associated protein 1 (Keap1) causes the release of a bound substrate, NF-E2-related factor 2 (Nrf2). This transcription factor then translocates to the nucleus, forms heterodimers with MAF proteins and activates HO-1 transcription from the MAF recognition element located in the gene promoter (reviewed by Ryter *et al.* 2006). Another factor, Bach1, forms heterodimers with Maf and competes with Nrf2/Maf dimers for binding to the stress-responsive element in the HO-1 gene promoter. However, unlike Nrf2, Bach proteins lack a transactivation domain, and consequently Bach/Maf dimers function as repressors of HO-1 transcription. The repressor activity of Bach1 overrules the activation by Nrf2, which is thought to explain the low levels of HO-1 activity at basal conditions. In fact, in the absence of Bach1, HO-1 is constitutively

expressed. It has been recently shown that transient increases in cellular haem are able to overcome this repression, as Bach1 binds with high affinity to haem and subsequently loses its DNA-binding activity (Ogawa *et al.* 2001; Ogawa *et al.* 2002; Sun *et al.* 2002). Bach1 function too seems to be regulated by the cell redox state. In this respect, oxidation of conserved cysteine residues in the DNA binding domain causes Bach1 to dissociate from the antioxidant response element, a mechanism by which ROS could release cells from Bach-1-mediated transcriptional repression (Ishikawa *et al.* 2005).

As mentioned above, the induction of HO-1 has been referred to as a cellular antioxidant defence mechanism. Reasons for this are: (i) the removal of free haem, which therefore becomes unavailable for participating in pro-oxidant reactions and (ii) the generation of the bile pigments biliverdin and bilirubin, which have antioxidant and anti-inflammatory properties, at least *in vitro*. However, the antioxidant effect of HO-1 cannot be explained by the replacement of haem with another potentially cytotoxic agent (iron). Indeed, it is possible that the iron released from haem by HO activity may enter a pool of intracellular "labile" or "chelatable" iron, which would be available for cellular iron-dependent processes; likewise, the iron released from HO activity would be transiently available for the promotion of intracellular ROS production. It has thus been suggested that HO activity may have pro-oxidant properties (Ryter and Tyrrell 2000). Evidence for the latter comes from studies where cells overexpressing HO-2 cultured in the presence of the enzyme substrate, hemin, showed increased levels of intracellular chelatable iron and hypersensitivity to an H_2O_2 challenge (Ryter and Tyrrell 2000). Likewise, cells overexpressing HO-2 were more sensitive to UVA, an effect that required pre-incubation with hemin and that was inhibited by DFO (Kvam *et al.* 2000). This is further supported by observations that treatments that cause induction of HO-1 activity are accompanied by a subsequent increase in ferritin synthesis (Vile and Tyrrell 1993). Nevertheless, whilst short exposures to hemin increase cell susceptibility to H_2O_2, long exposures (*e.g.* 16-20 hours) increase their resistance to the same challenge. In this respect, HO-1 induction is thought to be a major participant in the adaptive response of human skin fibroblasts to UVA. Upon exposure to UVA, HO-1 activity increases cellular ferritin, thereby rendering cells more resistant to a subsequent UVA exposure (Vile *et al.* 1994). It has been hypothesised that the

increase in ferritin synthesis caused by iron release from HO-1 activity may be an essential component of the cytoprotection afforded by HO-1 (discussed by Ryter *et al.* 2006).

1.4. Vitamin C

1.4.1. Dietary requirement of ascorbic acid

Descriptions of a disease associated with vitamin C deficiency, scurvy, became common during the extended sailing expeditions of the late 15th Century. The disease affected sailors deprived from fruits and vegetables for extended periods and caused larger personnel losses than those inflicted by battles and accidents (Hirschmannn and Raugi 1999). Despite ancient knowledge of the existence of an anti-scorbutic agent in fruits and vegetables, vitamin C was only isolated from vegetables in the twentieth century (Szent-Györgyi 1928). The vitamin was initially called "hexuronic acid," since it contained 6 carbons and was acidic. It was only a few years later that its anti-scorbutic properties were discovered and hence it was named as ascorbic acid (AA) (reviewed by Hirschmannn and Raugi 1999). AA is a water-soluble micronutrient required for multiple biological functions. It is as a cofactor for several enzymes participating in the post-translational hydroxylation of collagen, in the biosynthesis of carnitine, in the conversion of the neurotransmitter dopamine to norepinephrine, in peptide amidation and in tyrosine metabolism. In addition, AA is an important regulator of iron uptake. It reduces ferric to ferrous ions, thus promoting dietary non-haem iron absorption from the gastrointestinal tract, and stabilises iron-binding proteins. Higher vertebrates are able to synthesise vitamin C from D-glucose in the liver or kidney, but humans, other primates, guinea pigs and fruit bats lack the last enzyme involved in the synthesis of AA (gulonolactone oxidase) and so require the presence of the vitamin in their diet. In fact, the prolonged deprivation of vitamin C generates defects in the post-translational modification of collagen that cause scurvy and eventually death (Hirschmann and Raugi 1999; Halliwell 2001; Arrigoni and De Tullio 2002). The lack of the ability to synthesise ascorbate in primates is due to the accumulation of mutations in the gulonolactone oxidase gene, which are thought to have occurred approximately 70 million years ago (Nishikimi and Yagi 1991). The loss of gulonolactone oxidase activity during evolution may have initially occurred as an accident with no negative consequences in an environment which constantly supplied AA. However,

the fact that the lack of the enzyme became so widespread and then exclusive suggests that it represented an adaptive advantage. The enzyme catalyses the oxidation of gulonolactone by molecular oxygen and generates AA and H_2O_2 in equal proportions. H_2O_2 production, in turn, is circumvented by consumption of the intracellular antioxidant GSH. It has been argued that obtaining AA from food, rather than synthesising it, would be advantageous because it avoids H_2O_2 generation and consequently spares GSH levels (Banhegyi *et al.* 1997).

1.4.2. Redox metabolism of vitamin C

In addition to its anti-scorbutic action, AA is a potent reducing agent and a scavenger of free radicals in biological systems (reviewed in Rose and Bode 1993; Buettner and Jurkiewicz 1996; May 1999). Briefly, the mono-anion form (ascorbate) is the predominant chemical species at physiological pH. Ascorbate readily undergoes two consecutive, yet reversible, one-electron oxidations to generate dehydroascorbate (DHA) and an intermediate, the ascorbate free radical (AFR) (Figure 1.6). AFR is, however, a relatively unreactive free radical, with a reduction potential considerably lower than that of the α-tocopherol radical, the glutathione radical and virtually all reactive oxygen and nitrogen species that are thought to be involved in human disease (*e.g.* superoxide anion, hydroxyl radical, hydroperoxyl radical, singlet oxygen, nitrogen dioxide, nitroxide radicals and hypochlorous acid). These properties make ascorbate an efficient electron donor in many biological redox reactions, capable of replacing potentially highly damaging radicals by the poorly reactive ascorbate radical, according to equation 14 where AH^- represents ascorbate, $A^{\bullet-}$ represents the AFR and $X^\bullet$ represents the oxidising species:

$$AH^- + X^\bullet \longrightarrow A^{\bullet-} + XH \qquad [14]$$

Furthermore, ascorbate has the ability to recycle other important antioxidant molecules such as α-tocopherol and GSH from their respective radical species. Ascorbate can also be recycled by chemical and enzymatic mechanisms. AFR can be converted back to ascorbate by a NADH-dependent reductase or by dismutation of two molecules of the radical into one molecule of ascorbate and one molecule of DHA. DHA, in turn, is unstable at physiological pH and, unless it is reduced back to ascorbate, it may be irreversibly hydrolysed to 2,3-

diketogulonic acid and then oxidised to oxalic and threonic acid or catabolised through the pentose phosphate pathway (Banhegyi *et al.* 1997). Alternatively, DHA can be reduced back to ascorbate either directly by glutathione (May *et al.* 2001) or enzymaticaly by a glutathione-dependent DHA reductase, glutaredoxin, or the NADPH-dependent thioredoxin reductase (Wilson 2002).

1.4.3. Vitamin C bioavailability and transport

AA is water-soluble and is well absorbed from the gastrointestinal tract. Absorption occurs in the small intestine via a saturable, sodium-dependent active transport mechanism. Absorption efficiency of low oral doses of AA (4-64 mg) may be as high as 98 %, but decreases with increasing doses of the vitamin (Olson and Hodges 1987). Mean plasma AA levels are 50-60 μM for healthy, well-nourished, non-smoking individuals (*e.g.* Woollard *et al.* 2002; Astley *et al.* 2004). Plasma levels can be increased by long-term vegetarian diet (Szeto *et al.* 2004) and by oral supplementation up to approximately 100 μM (Woollard *et al.* 2002; Brennan *et al.* 2000; Choi *et al.* 2004). Thus, oral supplements can only modestly increase plasma AA concentration when comparing with the effect of a vitamin C-rich diet (Padayatty *et al.* 2004). The increase in plasma AA concentration as a function of supplement dose displays a sigmoid kinetics, with a steep increase between 30 and 100 mg/day and plasma saturation occurring at 1000 mg/day (Levine *et al.* 1996). Plasma saturation is due to efficient AA excretion in the urine (Levine *et al.* 1996). In fact, AA and

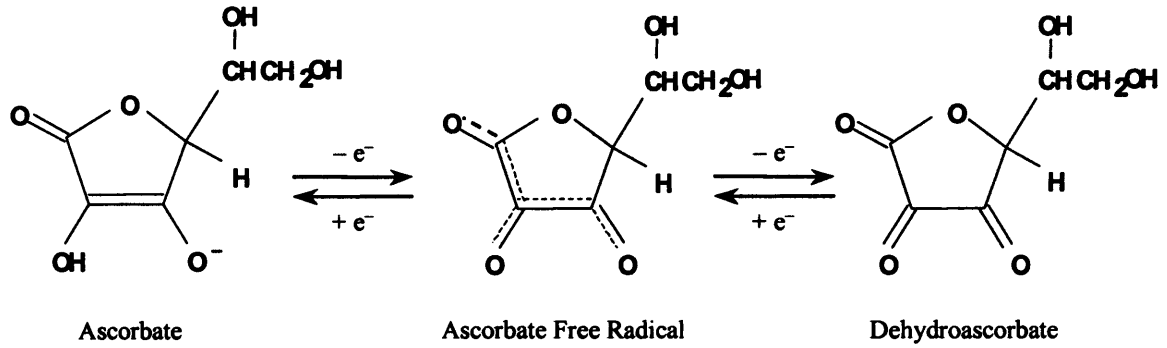


Figure 1.6. Redox metabolism of ascorbic acid. The one electron oxidation of ascorbate generates the ascorbate free radical, with one electron delocalised between three oxygen atoms, which on further oxidation originates dehydroascorbate. The latter is an unstable molecule and can be decomposed or reduced back to ascorbate.

its metabolites are excreted primarily in the urine, oxalate being normally the main excretion product. When plasma concentration is not higher than about 45 μM, AA is efficiently reabsorbed in the kidney and only oxalate is found in urine. However, renal re-absorption falls when plasma levels increase. At AA intakes above 90-100 mg/day re-absorption falls even faster and AA is excreted in the urine (Olson and Hodges 1987; Levine *et al.* 1996). Recent pharmacokinetics studies with human volunteers showed that, whilst plasma AA levels are tightly controlled when the vitamin is taken orally, intravenous administration can result in 70-fold higher plasma levels than the highest tolerated oral dose (3 g) (Padayatty *et al.* 2004).

Ascorbate is widely distributed in all tissues of the body, with higher levels found in the adrenal glands, pituitary and retina, and lower levels in kidney and muscle tissue. Tissue concentrations are usually 3-10 times higher than in the plasma (Olson and Hodges 1987). Some studies have shown that the increase in plasma AA was accompanied by an increase in the intracellular levels of the vitamin (*e.g.* Brennan *et al.* 2000); however, this increase is often not dose-dependent (Vojdani *et al.* 2000), which is due to cellular saturation. Thus it is known that the intracellular AA concentrations of neutrophils, monocytes and lymphocytes saturate at lower intake doses than human plasma (Levine *et al.* 1996).

Cellular AA transport has been studied *in vitro* and occurs by two distinct mechanisms. In the first mechanism, ascorbate enters mammalian cells via a family of specific transporters (SVCT1 and SVCT2) in a process driven by the sodium electrochemical gradient (Tsukaguchi *et al.* 1999). In the second mechanism, the oxidised form (DHA) is transported into the cells faster than the reduced form by facilitated diffusion through several isoforms of the glucose transporter (GLUT) (Vera *et al.* 1993; Diplock 1994; Rumsey *et al.* 1997), a process that can be inhibited by glucose in some but not all cell types (Korcok *et al.* 2003). Once inside the cells DHA is readily reduced back to AA by enzymatic mechanisms already mentioned, contributing to the intracellular accumulation of the reduced form. This recycling process allows many tissues to accumulate AA up to millimolar levels against a concentration gradient. It has been recently shown that the DHA reductase activity of cultured human keratinocytes can be

augmented in response to oxidative stress, simultaneously with an induction in catalase activity (Savini *et al.* 2003). The authors speculated that vitamin C recycling might be part of an adaptive response that renders cells more resistant to oxidants in general. It is likely that such an effect would be beneficial by sparing intracellular AA in cells undergoing oxidative stress, in which AA is being consumed and otherwise lost in an effort to neutralise ROS.

It is still unclear, however, which of the two mechanisms of AA transport predominates *in vivo*. Even though vitamin C seems to be present in human plasma mostly in the reduced form, a mechanism has been recently proposed to explain DHA uptake and subsequent intracellular vitamin C recycling by means of a bystander effect (Nualart *et al.* 2003). It was suggested that activated host-defence cells undergoing the oxidative burst promote oxidation of extracellular AA to DHA and this can be transported into neighbouring cells through the glucose transporters and immediately reduced back to AA.

1.4.4. Pro-oxidant effect of vitamin C

Paradoxically, vitamin C is also known to act as a pro-oxidant *in vitro*. The pro-oxidant properties of an $AA/Fe^{2+}/H_2O_2$ system have been recognized for a long time (Udenfriend *et al.* 1954). Mixtures of AA and iron or copper have been used for decades to induce oxidative modifications of lipids, proteins and DNA (Halliwell and Gutteridge 1999). AA contributes to oxidative damage formation by reducing ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions (equation 15) (and Cu^{2+} to Cu^{+}, as depicted in equation 16), which in turn can reduce H_2O_2 to hydroxyl radicals by Fenton chemistry (equations 4 and 17).

$$AH^- + Fe^{3+} \longrightarrow A^{\bullet -} + Fe^{2+} + H^+ \qquad [15]$$

$$AH^- + Cu^{2+} \longrightarrow A^{\bullet -} + Cu^{+} + H^+ \qquad [16]$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO^{\bullet -} \qquad [4]$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + OH^- + HO^{\bullet -} \qquad [17]$$

By favouring the cycle of repetitive reduction of ferric iron back to ferrous iron, ascorbate stimulates iron-induced lipid peroxidation *in vitro* (Miller and Aust 1989), in rat brain preparations (Sharma and Murti 1976) and in sickle erythrocyte membrane (Repka and Hebbel 1991). Ascorbate is also a major promoter of iron-catalysed hydroxyl radical formation in body fluids. In this respect, intravenous high-dose AA administration

increased the amount of loosely-bound iron detected by the BLM assay and elevated serum lipid peroxidation in iron-loaded guinea pigs (Kapsokefalou and Miller 2001). In addition, oral vitamin supplements enhance the rate of hydroxyl radical formation from H_2O_2 in human plasma when an iron catalyst (Fe^{2+}-EDTA) was present (Winterbourn 1981). In general, *in vitro* work has shown that AA has a pro-oxidant effect at low concentrations by recycling the iron catalyst in Fenton-like chemistry, whereas at high concentrations it acts as an antioxidant by scavenging hydroxyl radicals (Buettner and Jurkiewicz 1996). The biological significance of the *in vitro* or *ex vivo* pro-oxidant effect is, however, unclear. At physiological pH, *apo*-transferrin binds iron with great affinity and transferrin-bound iron is not available for participation in ascorbate-driven Fenton reactions (Aruoma and Halliwell 1987). Consequently, body fluids have little if any capacity to catalyse Fenton-type hydroxyl radical formation in the absence of an iron catalyst (Winterbourn 1981), hence it was argued that the pro-oxidant effect of AA may not be relevant *in vivo* (Halliwell 1999; Carr and Frei 1999). Nevertheless, transferrin saturation is observed in people with high iron levels or in pathological conditions associated with iron overload and catalytically active iron can be found in the plasma of these individuals (see Sections 1.3.2 and 1.3.3). Accordingly, AA is thought to enhance cell and organ damage in patients with iron overload. In this respect, several case reports in the literature have associated the consumption of high doses of AA with heart failure and death due to cardiac iron toxicity in individuals with idiopathic haemochromatosis and thalassemic patients with iron overload (McLaran *et al.* 1982; Nienhuis 1981; Herbert *et al.* 1993). Therefore, concerns that high-dose AA supplementation could accelerate destructive processes such as cancer and heart disease by increasing iron absorption and exacerbating iron-mediated tissue damage were raised (Olson and Hodges 1987; Mallory *et al.* 2003). In fact, it has been claimed that patients with primary or secondary iron overload should avoid AA supplements (Nienhuis 1981; Herbert 1993), despite their reduced levels of plasma (Young *et al.* 1994; Livrea *et al.* 1996) and white cell (Wapnick *et al.* 1968) ascorbate, which are thought to be a consequence of irreversible AA oxidation by iron.

Finally, it is also possible that not all the undesired effects of AA depend on the presence of transition metals. Indeed, a mechanism has been provided by which AA induces the decomposition of lipid hydroperoxides to genotoxic bifunctional electrophiles

in vitro without the need for free transition metal ions (Lee *et al.* 2001). It is still unknown, however, if this mechanism is relevant *in vivo*, and other authors have argued that in the more physiological context of human plasma incubated *in vitro*, AA prevents lipid peroxidation even in the presence of added redox-active transition metals and H_2O_2 (Suh *et al.* 2003).

1.4.5. Effects of vitamin C on iron metabolism

AA is capable of both chelating and reducing iron (Khan and Martell 1967) and there is some evidence that AA affects iron metabolism (Hoffman *et al.* 1991). First, AA is an important regulator of iron uptake. AA enhances dietary non-haem iron absorption from the gastrointestinal tract (Sayers *et al.* 1973; Cook *et al.* 1984) by converting insoluble Fe^{3+} to soluble Fe^{2+}, which is transported through the apical membrane of enterocytes by the divalent metal transporter 1 (McKie *et al.* 2002). Whether this means that AA supplements could lead to an increase in the body iron stores has not been established. Young *et al.* (1992) reported that high-dose oral AA supplementation increased plasma iron and hepatic iron stores in rats, but the same was not observed in humans (Sayers *et al.* 1973; Cook *et al.* 1984).

There is also evidence that AA enhances iron mobilisation *in vivo*. This is illustrated by the finding that AA increases urinary iron excretion in patients undergoing chelation therapy with DFO (O'Brien 1974). It was proposed that AA, due to its reducing properties, may release iron from ferritin, generating a pool of dissociable ferrous iron that is available for binding to a chelating agent such as DFO. The ability of AA to favour the release of catalytic iron from ferritin has been demonstrated *in vitro* (Tufano *et al.* 1981; Boyer and McCleary 1987). Moreover, oral AA supplementation raised the serum iron levels of South African Bantu subjects, who typically have high levels of iron accumulation in the tissues (Wapnick *et al.* 1970). It was suggested that AA causes the release of iron from intracellular iron stores into the serum. Accordingly, feeding guinea pigs an AA-free diet was shown to lower their serum iron levels (Gosiewska *et al.* 1996). Taken together, these data suggest that AA increases iron bioavailability.

1.4.6. Vitamin C in human intervention studies

The controversy around the *in vivo* anti- or pro-oxidant nature of vitamin C has been the subject of several human intervention studies in the recent years (reviewed by Duarte and Lunec 2005). The ability of AA to modulate oxidative DNA damage *in vivo* is of particular interest because some oxidative DNA lesions are thought to be pre-mutagenic (Wang *et al.* 1998). Therefore, many authors conducted trials in order to investigate the effect of AA consumption in biomarkers of oxidative DNA damage, including DNA base lesions and strand breaks. Most studies have looked at the effects of AA supplementation on oxidative damage to DNA of blood cells. The most popular base lesion has been 8-OHdG. The detection of 8-OHdG is relevant because not only is it one of the most abundant DNA lesions formed during oxidative stress, it is also mutagenic causing GC → TA transversions and it is implicated in carcinogenesis (Kasai 1997). In addition, it can be quantitatively measured as either the base product (8-OHG) or as the deoxynucleoside (8-OHdG) in tissues, serum and urine following exposure to oxidative stress with the use of high performance liquid chromatography with electrochemical detection (HPCL-EC), gas chromatography/mass spectrometry (GC/MS) or with antibody-based immunoassays (Collins *et al.* 2003). Unfortunately, a number of methodological difficulties have been associated with the measurement of this lesion. This is especially due to the artefactual oxidation of guanine in those methods that require DNA extraction and derivatisation, which tends to produce overestimated baseline levels of the lesion. The consequence was the formation of the European Standards Committee on Oxidative DNA Damage (ESCODD), which has aimed at establishing standard protocols and quality control steps during sample manipulation and ultimately reaching consensus over the basal level of DNA damage in human cells (Collins *et al.* 2000; 2003; Gedik *et al.* 2005).

DNA strand breaks measured by the single-cell gel electrophoresis (SCGE or comet assay) have been another popular biomarker in AA intervention studies. Although DNA SSBs are not a specific biomarker of oxidative stress, the specificity of the assay can be improved by including a digestion step with lesion-specific repair enzymes, namely formamidopyrimidine (FAPY) DNA glycosylase (FPG) and endonuclease III (Endo III), which recognise oxidised purines and pyrimidines, respectively. This modified version of the assay is able to detect low levels of damage with the advantage of avoiding artefactual

oxidation (Collins *et al.* 1993). Other studies have measured DNA single-strand breaks as an indirect indicator of antioxidant status. For this purpose, white blood cells have been challenged *ex vivo* with a DNA strand breaking oxidant (usually H_2O_2 or ionising radiation) to assess the donor's antioxidant status, assuming that the intracellular antioxidants would prevent DNA breakage.

A comprehensive list of intervention studies where DNA damage was measured as either nucleotide base lesion or DNA strand breakage is supplied in Table 1.1. This includes studies performed to date in which AA has been administered to healthy or diseased individuals as a dietary supplement or intravenously, either alone or in combination with other antioxidants. Also, it includes studies of the effects of a single vitamin administration and those where individuals were supplemented for periods of several weeks or months.

Regarding the measurement of DNA base lesions, an early report has shown that AA content in the semen was inversely related to the level of 8-OHdG in sperm DNA (Fraga *et al.* 1991) and more recently it was reported that 8-OHdG in lymphocyte DNA from human healthy volunteers was negatively correlated with the intracellular AA levels (Lenton *et al.* 1999). But the question still remained of whether AA supplements could contribute to reduce the levels of the lesion *in vivo*. Podmore *et al.* (1998a) supplemented healthy individuals with 500 mg AA for 6 weeks and observed a decrease in 8-OHG in lymphocyte DNA relative to both placebo and baseline levels, suggesting that AA was acting directly as an antioxidant *in vivo*. However, the authors found a simultaneous increase in the level of another base oxidation product, 8-oxoadenine (8-OHA), suggesting a concomitant pro-oxidant effect. 8-OHA is, however, much less mutagenic than 8-OHG, so the authors argued that the study had shown an overall protective effect of AA (Podmore *et al.* 1998b). In a later publication, but as part of the same study, Cooke *et al.* (1998) found significant decreases in DNA levels of 8-OHdG that strongly correlated with increases in plasma ascorbate concentration *in vivo*, and reported significant subsequent increases in serum and urinary 8-OHdG levels, which could be products of DNA repair. The authors suggested that AA did not inhibit 8-OHdG formation but rather promoted its removal. It was hypothesised that AA would initially cause oxidative DNA damage via a pro-oxidant activity and consequently cause the up-regulation of DNA repair processes that promote the

Table 1.1. Human intervention studies relating vitamin C supplementation with biomarkers of oxidative DNA damage

Study description	Measured endpoint	Effect	Reference
10 men initially kept on a controlled diet (250 mg/day) were subsequently given depletion (5 mg/day) and repletion (250 or 60 mg/day) doses for periods of 1 month	8-OHdG in sperm and lymphocyte DNA and 8-OHG in urine by HPLC-EC or HPLC-UV	Increase in 8-OHdG levels following the ascorbate depletion that was reduced upon repletion of dietary ascorbate; no effect on urine and lymphocyte DNA	Fraga *et al.* 1991; Jacob et al. 1991
6 healthy individuals who had fasted overnight were given a single 500 mg dose with breakfast; blood samples were taken immediately before and 1 hour later	DNA breaks in white blood cells with or without ex-vivo ionising radiation challenge by SCGE	Decrease in endogenous and radiation-induced DNA strand breaks	Green *et al.* 1994
100 healthy males (smokers and non-smokers) were randomly assigned to receive 100 mg/day in combination with vitamin E (280 mg) and β–carotene (25 mg) or placebo for 20 weeks	DNA breaks and oxidised pyrimidine bases in lymphocytes with and without ex-vivo H_2O_2 challenge by SCGE	Decrease in oxidised pyrimidines (but not in strand breaks) in smokers and in non-smokers; decrease in strand breaks produced by ex-vivo exposure to H_2O_2	Duthie *et al.* 1996
12 individuals (smokers and non-smokers) were given a single 1g dose	DNA breaks in lymphocytes with ex-vivo H_2O_2 challenge by SCGE	Decrease in H_2O_2-induced DNA breaks in smokers and in non-smokers 6 hours after vitamin supplementation	Panayiotidis and Collins 1997
48 individuals with a range of serum cholesterol levels were assigned to and cycled through one of the supplements (60 or 6000 mg/day) and placebo for 2 weeks each, all separated by 6-week washout periods	DNA breaks in lymphocytes with or without ex-vivo H_2O_2 challenge by SCGE	No effect on endogenous or ex-vivo H_2O_2-induced levels of DNA breaks	Anderson *et al.* 1997
6 healthy smokers were randomly assigned to receive 500 mg/day or placebo for 4 weeks; 5 healthy non-smokers were used as a control	8-OHdG in PBMC by HPLC-EC	Decrease in 8-OHdG levels (but not statistically significant)	Lee *et al.* 1998
30 healthy individuals were on placebo for 6 weeks and then were given 500 mg/day for the next 6 weeks	8-OHA and 8-OHG in lymphocytes by GC/MS and HPLC-EC; 8-OHdG in serum and urine by immunoassay	Increase in 8-OHA and decrease in 8-OHG levels followed by increases in serum and urine 8-OHdG	Podmore *et al.* 1998a; Cooke *et al.* 1998

38 healthy individuals stratified by plasma ascorbate levels given 60 or 260 mg/day in combination with ferrous sulphate (14 mg/day) for 12 weeks	Twelve different oxidative base lesions in DNA isolated from whole blood by GC/MS	Increase in 5-oxomethylhydantoin, 5-oxohydantoin and FAPY guanine at 6 weeks and decrease in 8-OHG at 12 weeks in individuals with higher initial plasma ascorbate	Rehman *et al.* 1998
30 individuals exposed to environmental tobacco smoke were given 60 mg/day in combination with β-carotene (3 mg), vitamin E (30 I.U.), zinc (40 mg), selenium (40 ug) and copper (2 mg) for 8 weeks; 36 non-exposed individuals were used as a control	8-OHdG in DNA isolated from whole blood by HPLC-EC	Exposed individuals had significantly higher levels of 8-OHdG than the control (non-exposed) group, but these decreased to levels below those in the control group upon antioxidant supplementation	Howard *et al.* 1998
21 healthy individuals (9 smokers and 12 non-smokers) were randomly assigned to and cycled through one of the supplements (350 mg/day alone or in combination with 250 mg vitamin E for 4 weeks) and placebo (consisting of a 8-week washout period)	8-OHdG in mononuclear leukocyte DNA by HPLC with colorimetric detection and DNA breaks with or without ex-vivo X-ray challenge by SCGE	No effect on endogenous and radiation-induced DNA strand breaks in smokers and in non-smokers; decrease in 8-OHdG levels in non-smokers (but not statistically significant)	Welch *et al.* 1999
20 healthy individuals were randomly assigned to and cycled through one of the supplements (260 mg/day alone or in combination with 14 mg/day ferrous sulphate) and placebo for 6 weeks, all separated by 8-week washout	Twelve different oxidative lesions in DNA isolated from whole blood by GC/MS	Decrease in 8-OHG and 5-oxomethyluracil in both treatments and increase in 5-oxomethylhydantoin and 5-oxocytosine in ascorbate + iron, but all changes also present in the placebo	Proteggente *et al.* 2000
111 healthy smokers were randomly assigned to receive 500 mg/day in combination with vitamin E (200 IU) and β-carotene (6 mg) or placebo for 6 months	8-OHdG in oral cells and mononuclear cells by immunoperoxidase staining	Decrease in 8-OHdG levels in both treated and placebo groups	Jacobson *et al.* 2000
15 SLE female patients were on placebo during the 6 weeks and were given 500 mg/day for the next 6 weeks	8-OHdG in lymphocytes by HPLC-EC and in serum and urine by immunoassay	No effect on 8-OHdG levels in DNA or urine, increase in serum 8-OHdG	Evans *et al.* 2000
7 healthy individuals given 1 g/day for 6 weeks	DNA breaks in lymphocytes with and without ex-vivo	Decrease in H_2O_2-induced (but not in endogenous) DNA damage	Brennan *et al.* 2000

	H_2O_2 challenge by immunoassay		
20 healthy individuals were randomly assigned to receive either 500, 1000 or 5000 mg/day or placebo for 2 weeks	8-OHG in lymphocytes by HPLC-EC	Decrease in 8-OHG only in the group supplemented with 1000 mg/day	Vojdani *et al.* 2000
30 asymptomatic HIV-seropositive patients were randomly assigned to receive 50 mg/day in combination with vitamin A (5000 units) and vitamin E (100 units) or placebo for 6 months	8-OHG, 5-oxouracil and 5-oxocytosine in lymphocyte by GC/MS	Decrease in 8-oxoguanine and 5-oxouracil levels	Jaruga *et al.* 2002
40 healthy individuals received placebo during the 5 weeks and then received 400 mg/day for 16 weeks	gdC in PBMC DNA by enzyme-linked immunosorbent assay	Increase in gdC levels at 6-16 weeks and a subsequent decrease relative to baseline on continued supplementation	Cooke *et al.* 2003
9 healthy men were given 60 mg every other day for 3 weeks	DNA breaks and DNA repair capacity in lymphocytes by SCGE and cell-free DNA repair assay, respectively	No effect on baseline or ex-vivo H_2O_2-induced levels of DNA breaks or on DNA repair capacity	Astley *et al.* 2004
12 healthy individuals were randomly assigned to and cycled through one of the supplements (single 500 mg dose alone or in combination with 400 units of vitamin E) and placebo, all separated by at least 10 days washout	DNA breaks in lymphocytes with or without ex-vivo H_2O_2 challenge by SCGE	No effect on endogenous or H_2O_2-induced DNA breaks 90 or 180 minutes after supplementation	Choi *et al.* 2004
51 chronic haemodialysis patients were randomly assigned to receive intravenous 300 mg/day or placebo post-dialysis 3 times/week for 8 weeks	8-OHdG in peripheral blood lymphocytes by HPLC-EC	Decrease in 8-OHdG level relative to baseline and placebo group	Tarng *et al.* 2004
48 smokers were randomly assigned to receive either plain release or slow release tablets corresponding to 500 mg/day in combination with vitamin E (182 mg) or placebo for 4 weeks	DNA breaks and oxidised bases in mononuclear blood cells determined as EndoIII or Fpg sensitive sites with the SCGE	Decreased levels of EndoIII and FPG sensitive sites at 4h, 8h and 4 weeks following supplementation with slow release tablets, whereas plain release tablets only decreased EndoIII sites at 4h and 8h. No effect on endogenous DNA breaks.	Moller *et al.* 2004

n.a., not available

removal of highly mutagenic lesions. More recently, Cooke *et al.* (2003) reported that AA supplementation increased the levels of deoxycytidine glyoxal (gdC), a putative product of lipid peroxidation and auto-oxidation of AA and glucose, which was also suggestive of a pro-oxidant effect *in vivo*. The levels of gdC were, however, significantly reduced upon continued vitamin supplementation, suggesting once again that AA may promote lesion removal by up-regulating repair processes.

Further evidence for a pro-oxidant effect of AA *in vivo* came from Rehman *et al.* (1998). The authors observed a significant rise in several oxidative DNA base damage products (5-OH methylhydantoin, 5-OH hydantoin and FAPY guanine) in the white blood cells of healthy human volunteers with a high initial plasma AA concentration after 6 weeks of co-supplementation with iron and AA. On the other hand, levels of 8-oxo-G decreased following 12 weeks of supplementation. In individuals with lower initial levels of plasma AA, pre-supplemental levels of oxidative DNA damage were higher and decreased on supplementation. These studies were, however, criticised for not including a true placebo control (Poulsen *et al.* 1998). In fact, the trial reported by Podmore *et al.* (1998a) was sequential, with the placebo period preceding the AA supplementation period, whereas the study by Rehman *et al.* (1998) did not include a placebo group at all, leaving a possibility of false positive results caused by seasonal variation or changes in food habits and lifestyle of the subjects. In fact, when the same authors attempted to reproduce the latter study by including a more appropriate placebo control group, no increase in oxidative DNA lesions was found following AA supplementation alone or in combination with iron (Proteggente *et al.* 2000). It is noteworthy that, as in their previous study, AA supplementation decreased the levels of 8-OHG when compared with pre-supplemental levels; however, a similar decrease in 8-OHG was observed in the placebo group. This observation reinforces the importance of including a placebo-controlled, parallel design rather than a sequential study design.

In two other placebo-controlled supplementation trials with healthy individuals, Welch *et al.* (1999) claimed that AA supplementation alone or in combination with vitamin E for 4 weeks did not have an effect on oxidative damage, but their results show a 50 % reduction in leukocyte 8-OHdG, while Vojdani *et al.* (2000) observed a reduction in

lymphocyte 8-OHdG following AA supplementation with 1000 mg per day for 2 weeks. In addition, four placebo-controlled supplementation trials were performed with smokers, a condition that is known to be associated with increased oxidative damage to DNA. Lee *et al.* (1998) supplemented smokers with 500 mg AA per day for 4 weeks and observed a decrease in 8-OHdG levels in white blood cells, even though this effect was not statistically significant. Jacobson *et al.* (2000), on the other hand, supplemented smokers with AA in combination with vitamin E and β-carotene and reported a decrease in 8-OHdG levels both in treated and in placebo groups. But a combination of the same antioxidants was able to decrease the endogenous levels of oxidised pyrimidines (assessed as Endo III sensitive sites with the use of the comet assay) in lymphocyte DNA of smokers and non-smokers (Duthie *et al.* 1996). Recently, Moller *et al.* (2004) also reported that oral supplementation with 500 mg AA in combination with 182 mg vitamin E per day protected blood mononuclear cells of smokers against oxidative DNA damage by decreasing the amount of Endo III and FPG sensitive sites. Interestingly, the protective effect was only evident for a few hours after ingestion when the AA was supplied as plain release tablets; however, supplementation with slow release tablets afforded a longer-term protection that was still evident at the end of the trial (4 weeks). In a different study, whole blood DNA from individuals exposed to environmental tobacco smoke contained increased levels of 8-OHdG, which decreased to levels below those in the control group on supplementation with an antioxidant cocktail containing AA (Howard *et al.* 1998). Finally, three trials have assessed the effect of AA supplementation on DNA base oxidation in other conditions that have been associated with oxidative stress. Evans *et al.* (2000) reported no effect of AA supplementation on lymphocyte DNA 8-OHdG of systemic lupus erythematosus (SLE) patients. Human immunodeficiency virus (HIV)-infected patients had significantly higher levels of two oxidised DNA bases (8-OHG and 5-OHUra) that were decreased upon AA supplementation in combination with vitamins A and E (Jaruga *et al.* 2002). More recently, a randomised, placebo-controlled study with chronic haemodialysis patients showed additional evidence for a role of AA in promoting removal of 8-OHdG. In these subjects, AA intravenous supplementation for 8 weeks was able to reduce the lymphocyte 8-OHdG levels (Tarng *et al.* 2004).

Regarding the measurement of DNA strand breaks, seven out of eight studies showed a null effect (Table 1.1). Hence there is no evidence that AA supplementation can alter the endogenous levels of DNA strand breakage. Results from studies where white blood cells were collected from patients and subsequently challenged *ex vivo* with an oxidant insult are conflicting. Three studies investigated the effect of a single-dose AA supplementation. Green *et al.* (1994) reported a protection against *ex vivo* exposure to ionising radiation that started as early as 1 hour after a single AA ingestion (500 mg) with breakfast and peaked at 4 hours. However, breakfast itself had a protective effect. Panayiotidis and Collins (1997) reported that a single high dose (1 g) AA supplement afforded protection against oxidative DNA damage caused by *ex vivo* exposure to H_2O_2. In agreement with the study of Green *et al.* (1994), this protection peaked at 2-4 hours after the ingestion. However, a recent study failed to show any effect of a single dose of AA alone, or in combination with vitamin E, on resistance to an *ex vivo* oxidative challenge, despite the clear increase in plasma AA (Choi *et al.* 2004). Other trials have investigated the effects of long-term AA supplementation. Antioxidant supplementation with a combination of AA, vitamin E and β-carotene for 20 weeks decreased the damage induced when lymphocytes were challenged *ex vivo* with H_2O_2 (Duthie *et al.* 1996). Brennan *et al.* (2000) supplemented individuals with AA on its own and observed a similar protective effect, which correlated with increases in patient lymphocyte intracellular AA levels. However, other studies have failed to show a protective effect (Anderson *et al.* 1997; Welch *et al.* 1999; Astley *et al.* 2004).

To summarise, more than twenty studies have been carried out so far to determine the effect of AA supplementation on biomarkers of oxidative DNA damage in humans. It is important to note that most studies have measured levels of 8-OHdG using chromatographic methods that are known to lead to spurious base oxidation, so results need to be interpreted with caution (Gedik *et al.* 2005). Nevertheless, most of the studies showed either an AA-mediated reduction in oxidative DNA damage or a null effect. The studies involving the measurement of base lesions have generally supported the idea that levels of 8-OHdG are negatively correlated with ascorbate plasma concentration, whereas a few controversial studies measuring other base lesions have shown an increase in DNA oxidation following AA supplementation. The latter ones suggest that the pro-oxidant

effect may occur *in vivo* but this hypothesis remains unclear and more studies are required before a conclusion is reached.

A possible reason for the discrepancies encountered between different studies might be the AA intracellular saturation. It is known that blood cells saturate at lower AA concentrations than human plasma and that this intracellular saturation occurs at plasma concentrations that can easily be obtained from the diet (Levine *et al.* 1996). However, most studies only report increases in plasma levels. It is likely that if tissue saturation is achieved, then the additional beneficial effects are small and difficult to detect, leading to non-significant or null effects. Consistent with this notion, most of the studies that have shown a protective effect were carried out with smokers or patients with pathological conditions associated with oxidative stress and low plasma AA levels. It is thus possible that the initial level of AA in the cell predetermines whether supplementation trials may have a positive or null response.

1.4.7. Vitamin C in human disease

Low levels of plasma AA are commonly associated with conditions of increased oxidative stress, such as rheumatoid arthritis (Jaswal *et al.* 2003), cataracts (Simon and Hudes 1999), HIV infection (Jaruga *et al.* 2002), SLE (Evans *et al.* 2000), diabetes mellitus (Cunningham 1998), cancer and smoking habits (Polidori *et al.* 2001). The possible use of AA in cancer therapy and prevention has been an area of great interest. It is tempting to speculate that AA supplements, if able to prevent the formation and/or promote the repair of pre-mutagenic oxidative DNA lesions, could be of use in cancer prevention. An early report showed that daily supplementation with AA at high doses (grams) increased the survival time of terminal cancer patients (Cameron and Pauling 1976) and it was suggested that AA could have important anticancer properties (Cameron *et al.* 1979). Indeed, AA kills or inhibits growth of many tumour cell lines (reviewed by Alcain and Buron 1994) and potentiates the cytotoxicity of radiosensitising drugs (Koch *et al.* 1979). There are also several reports showing that cancer cell lines are more sensitive to AA than their non-malignant counterparts (*e.g.* Prasad *et al.* 1979; Park *et al.* 1980). However, so far only a limited number of studies have established an association between AA administration and survival of advanced cancer patients and these have been highly criticised for the lack of

appropriate controls and randomisation (Block and Mead 2003). Regarding cancer prevention, several epidemiological studies have linked the consumption of a diet rich in fruit and vegetables (and therefore in antioxidants) with lower incidence of many types of cancer (reviewed by Steinmetz and Potter 1996). Recently, Bjelakovic *et al.* (2004) presented the results of their systematic review of all randomised trials relating antioxidant supplements with the incidence of gastrointestinal cancers, which included meta-analyses of outcomes such as cancer incidence and mortality. The authors found no evidence that antioxidants can prevent gastrointestinal cancers. On the contrary, certain antioxidant combinations (β-carotene with vitamin A or vitamin E) result in increased patient mortality. AA, when added alone or in combination with other antioxidants, did not seem to have an effect on the incidence of gastrointestinal cancers or on overall mortality.

Epidemiological evidence has also associated fruit and vegetable consumption with lower risk of cardiovascular disease (CVD) (Bazzano *et al.* 2002). Notably, low plasma levels of AA were associated with death from CVD (Khaw *et al.* 2001) and it has been speculated in the literature that AA may protect against CVD through several mechanisms. AA enhances endothelium-dependent vasodilatation, thereby preventing endothelial dysfunction associated with atherosclerosis, hypercholesterolemia, hypertension, diabetes and smoking. This process seems to involve the ability of AA to increase the atheroprotective nitric oxide (May 2000). AA was shown to enhance the activity of endothelial NO synthase by keeping its cofactor, tetrahydrobiopterin, in a reduced state and thereby increasing its intracellular availability (Huang *et al.* 2000; Heller and Werner 2002). In addition, AA prevents oxidation of low-density lipoproteins (LDL), a critical process during atherosclerosis and cardiovascular disease (Retsky *et al.* 1993), and decreases damage caused by oxidised LDL to endothelial cells. Indeed, pre-treatment of cultured human arterial smooth muscle cells with AA protected against apoptotic cell death induced by oxidised LDL (Siow *et al.* 1999). It was speculated that, by protecting against vascular cell death, AA could limit plaque instability in advanced atherosclerosis and consequently protect against thrombosis. AA has also an anti-inflammatory action in decreasing leukocyte adhesion to the endothelium. Thus individuals with low plasma AA levels have greater monocyte adhesion to endothelial cells (Woollard *et al.* 2002) and express higher levels of monocyte intracellular adhesion molecule 1 (ICAM-1) mRNA

(Rayment *et al.* 2003), but supplementation with 250 mg AA/day for 6 weeks was able to reduce monocyte adhesion and ICAM-1 expression. However, despite all the proposed mechanisms and the epidemiological observations, data from clinical trials relating AA with different CVD endpoints were inconsistent (Loria 2002) and there is still no clear evidence that AA (as well as other dietary antioxidants) may prevent CVD (Marchioli 1999).

In summary, results from several epidemiological studies associate low levels of plasma AA with increased death from CVD and cancer. However, whilst it seems likely that an increase in consumption of antioxidant-rich foods such as fruit and vegetables would offer some degree of protection, clinical trial data are not conclusive as to whether AA supplements are beneficial in well-nourished individuals.

1.4.8. Effects of vitamin C on cell culture models

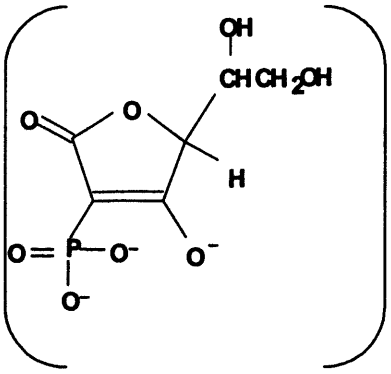
As mentioned above, recent studies with cultured cells have helped to elucidate the mechanisms of AA cellular uptake (see Section 1.4.3). However, other effects of AA have been elucidated in the past using cell culture models, especially human diploid fibroblasts (HDFs). There is ample evidence that AA stimulates collagen synthesis in HDFs and this is related to its ability to act as a co-factor in the hydroxylation of proline (Blanck and Peterkofsky 1975; Freiberger *et al.* 1980; Murad *et al.* 1981). In addition, AA is known to modulate cell proliferation. In this respect, AA stimulates the growth of HDFs in culture when supplied at confluent density (Hata *et al.* 1988; Chan *et al.* 1990; Chepda *et al.* 2001a), whereas at low cell density or at high (millimolar) concentrations it becomes cytotoxic (Peterkofsky and Prather 1977; Michiels *et al.* 1990; Peterszegi *et al.* 2002). The cytotoxicity is mediated by H_2O_2 formation in the extracellular environment (Peterkofsky and Prather 1977) and is thought to be associated with the auto-oxidation of AA in the culture medium. In fact, it has been known for a long time that the oxidation of AA in the presence of Fe^{2+} and oxygen leads to the formation of H_2O_2 and causes the oxidation of other substrates (Udenfriend *et al.* 1954). In the presence of oxygen, AA auto-oxidises and is rapidly lost from aqueous solutions (including salt and buffer solutions and cell culture media) at physiological temperature and pH (Winkler 1987). The oxidation of AA is apparently due to the presence of trace levels of catalytic transition metals in most salt and

buffer solutions employed in research (Buettner 1988). According to the author, iron and copper are commonly present in these solutions at trace concentrations of 1-10 μM and < 0.1 μM, respectively. When chelating agents were used to render solutions free of these catalytic metals, AA was stable even at a neutral pH. Likewise, the loss of endogenous AA from human plasma is accelerated in the presence of added iron (Suh *et al.* 2003).

Importantly, the incubation of cell culture medium with either sodium ascorbate or L-ascorbic acid leads to the formation of measurable amounts of H_2O_2 (Clément *et al.* 2001; Wee *et al.* 2003). In fact, it has been suggested that many of the effects of AA on cultured cells may be due to the H_2O_2 formed by interaction of AA with the cell culture medium (Halliwell *et al.* 2000). Whilst H_2O_2 itself is a poorly reactive molecule, in the presence of transition metal ions it can be converted to highly reactive hydroxyl radicals by the Fenton reaction (see Section 1.3.3). In fact, the cytotoxicity of AA is enhanced with the addition of metal ions (Satoh and Sakagami 1997) and prevented by CAT, as observed in mouse neuroblastoma cells and in HDFs (Peterkofsky and Prather 1977; Prasad *et al.* 1979; Peterszegi *et al.* 2002). High (millimolar) doses of sodium ascorbate have also caused an increase in sister-chromatid exchanges and cytotoxicity, and the inhibition of DNA synthesis in cultured cells, effects that were repressed or reduced by CAT (Galloway and Painter 1979). Likewise, millimolar (non-physiological) levels of AA cause redifferentiation and growth inhibition in human hepatoma cells and it has been suggested that this effect may be due to an increase in the H_2O_2 content of these cells (Zheng *et al.* 2002). Moreover, the ability of AA to selectively suppress the growth of several tumour cell lines can at least in some cases be explained by the production of H_2O_2 in the medium (*e.g.* Park *et al.* 2004; Menon *et al.* 1998). The toxicity of AA towards cancer cells may be a consequence of their low levels of endogenous CAT activity (Alcain and Buron 1994). The involvement of H_2O_2 may also explain the controversy around some reports describing the effects of AA on cell death by apoptosis. AA protects some cell types from apoptotic cell death induced by serum withdrawal (Barroso *et al.* 1997) or by the apoptotic agents 6α-methylprednisolone, thapsigargin and etoposide (Maellaro *et al.* 1996). In the latter study, protection was also induced by incubation with H_2O_2 and suppressed if CAT was present during incubation with AA, indicating that the effects of AA were mediated by H_2O_2 originating from its auto-oxidation. In other experiments, unstable vitamin C derivatives

(L-ascorbic acid or sodium ascorbate) were found to induce apoptosis in human promyelocytic leukemic HL60 cells, whereas vitamin C derivatives that do not autoxidise such as L-ascorbic acid-2 phosphate (AA2P) magnesium salt and L-ascorbic acid 2-sulfate did not have any apoptotic activity (Sakagami *et al.* 1996). Moreover, apoptosis was abolished in the presence of CAT, showing that AA toxicity was due to extracellular H_2O_2 production (Park *et al.* 2004; Clement *et al.* 2001). In addition to cell death, AA was also reported to enhance the differentiation of HL-60 cells to granulocytes and monocytes (Alcain and Buron 1994), presumably by modifying the cellular redox state of differentiating cells. Again, these effects were not achieved when using DHA or a stable AA derivative (Lopez-Lluch *et al.* 2001) and the differentiation was abolished in the presence of CAT, showing that it was a result of H_2O_2 formation in the medium (Kang *et al.* 2003). Some authors have also shown that AA effects are not due to a pH change in the medium at least when using AA concentrations up to 1 mM (Takahashi *et al.* 2003; Park *et al.* 2004).

As a way to circumvent the problems associated with AA auto-oxidation, some authors have used AA2P (Figure 1.7), a vitamin C derivative that remains stable in cell culture medium even after several days of incubation at 37 °C until it is hydrolysed by cellular phosphatases to AA and inorganic phosphate (Hata *et al.* 1989). AA2P is taken up from the medium and accumulated intracellularly as AA against a concentration gradient (Savini *et al.* 1999). In human vascular endothelial (HUVE) cells, AA2P leads to a higher intracellular enrichment in ascorbate than equimolar concentrations of AA (Furumoto *et al.* 1998). AA2P is also superior to AA in enhancing proliferation, collagen accumulation and extracellular matrix formation in cultured HDFs (Hata *et al.* 1989). In addition, AA2P can be useful in cell culture due to its efficiency in stabilising α-tocopherol when both are added to cells in culture medium (Chepda *et al.* 2001b). Also, some evidence exists that AA2P may be superior to AA as an antioxidant supplement to cells in culture. Presumably by increasing the cellular antioxidant capacity, AA2P protects human keratinocytes from UVB-induced cell death (Kanatate *et al.* 1995; Savini *et al.* 1999) and primary cell lines from *in vitro* cellular ageing (Furumoto *et al.* 1998; Kashino *et al.* 2003). It is known that primary mammalian cells can undergo only a limited number of cell divisions when cultured in the laboratory, before reaching a non-proliferating G_0 senescent state. This loss



3/2 Mg^{2+}

Figure 1.7. Molecular structure of magnesium L-ascorbate 2-phosphate (AA2P).

of replicative potential is associated with telomere shortening and also with increased sensitivity to oxidative stress (de Magalhaes 2004). Indeed, oxidative stress has been implicated in the process of ageing and it is known that primary HDFs grown in the presence of high oxygen concentrations have a reduced lifespan and an increased rate of telomere shortening (reviewed in Finkel and Holbrook 2000). Notably, AA2P reduced the rate of telomere shortening during the cellular ageing process of HUVE cells and HDFs and extended their replicative lifespan (Furumoto *et al.* 1998; Kashino *et al.* 2003). This effect was attributed to its antioxidant properties, since AA2P decreased the level of intracellular ROS of control replicating cells as judged by a reduction in 2',7'-dichlorofluorescein diacetate fluorescence.

Finally, it is known that AA causes strand breaks to DNA molecules in solution in the presence of oxygen (Bode 1967) and this action is suppressed by CAT, which indicates that H_2O_2 is involved (Morgan *et al.* 1976). Likewise, *in vitro* AA causes hydroxylation of the C-8 position of deoxyguanosine in the presence of oxygen (Kasai and Nishimura 1984). Two studies employing the alkaline comet assay have shown that, in some but not all conditions, AA causes DNA strand breakage in cultured cells (Anderson *et al.* 1994; Singh 1997).

1.4.9. Effects of vitamin C on gene expression

It is well known that ROS can act as subcellular messengers in several gene regulatory and signal transduction pathways, so it is not surprising that antioxidants, as well as oxidants, are able to activate certain genes and signalling pathways by modulating the redox state of the cell (Allen and Tresini 2000). In addition, the binding activity of certain

transcription factors is determined by the redox state of the cell. NFkB and AP-1, for example, are well-known mediators of redox-responsive gene expression (Zhou *et al.* 2001). ROS can activate NFkB presumably by causing release of the inhibitory subunit (IkB) from the NFkB complex, whereas redox regulation of activating protein 1 (AP-1) binding may occur through a conserved cysteine residue present in its Jun and Fos subunits (Allen and Tresini 2000).

In some cell culture conditions it has been shown that AA, due to its pro-oxidant or antioxidant properties, can modulate nuclear binding of redox sensitive transcription factors such as AP-1 and NF-k-B. For example, AA potentiates the phorbol 12-myristate 13-acetate (PMA)-induced AP-1 binding to DNA in murine macrophages (Arkan *et al.* 2001). AA2P, in turn, induces resistance of skeletal muscle cells to oxidative stress by modulating the binding of NFkB and AP-1 complexes, namely by increasing activity of the former and by inhibiting the latter (Catani *et al.* 2004).

Another possible mechanism by which AA may modulate gene expression is related to its intracellular recycling process. A model has been proposed to explain the pro-oxidant effect of AA in neuronal cells in which AA is autoxidised extracellularly to DHA and the latter is rapidly transported into the cells by the GLUT transporters. Once in the cytosol, DHA is reduced back to ascorbate and this reduction process is thought to cause the oxidation of cellular components (Song *et al.* 1999; 2001). It is thus possible that AA recycling inside the cells could lead to the formation of intracellular ROS and consequently interfere with redox-sensitive signalling pathways, eventually inducing gene expression (Griffiths and Lunec 2001). Even though the effects of AA on gene expression are of particular interest, only recently have they gained more attention. Catani *et al.* (2001) studied expression profiles of transformed human epidermal keratinocytes (HaCaT) exposed to a high dose of AA2P (1 mM) for 5 hours using cDNA array technology. AA2P increased the expression of Fra-1, GST-pi and Mut L homologue-1 (MLH1). Fra-1 is a member of the Fos superfamily, which heterodimerises with members of the Jun family and acts as a negative regulator of AP-1 activity. When HaCaT cells were irradiated with UVB, pre-incubation with AA2P was able to modulate the binding of the transcription factor AP-1. This was achieved in part by changing the composition of the AP-1 complex in irradiated cells through an increase in the steady-state levels of Fra-1 protein and also by decreasing

activation of the stress-activated JNK and consequently c-Jun phosphorylation. In a subsequent publication, the authors have confirmed that AA2P induces expression of MLH1, a member of the DNA mismatch repair machinery. In addition, AA2P specifically induced p73, an apoptosis-inducing protein that is a target of MLH1 and increases cellular susceptibility to apoptosis in response to cisplatin. The authors speculated that ascorbate, by inducing gene expression of MLH1 and p73, potentiates tumour cell susceptibility to apoptotic death by cisplatin, which in turn could explain its possible chemopreventive activity (Catani *et al.* 2002). A similar effect was observed in a different study, where cervical carcinoma HeLa cells were sensitised to apoptotic cell death induced by cisplatin or etoposide by pre-loading with a low, non-toxic concentration of AA (1 μM) (Reddy *et al.* 2001). In this work the effect was apparently associated with down-regulation of c-Jun and c-Fos, up-regulation of p53 and increased Bax/Bcl2 ratio.

Other authors recently looked at the effect of a pro-oxidant mixture of iron and a high (millimolar) dose of AA on gene expression of intestinal epithelial cells (Bernotti *et al.* 2003). Not surprisingly, these workers observed an increase in lipid peroxidation that was accompanied by the activation of transcription factor NFkB and increased expression of the inflammatory proteins ICAM-1, cyclooxygenase-2 and interleukin-8.

Recently, the effects of AA on gene expression have also been studied in the context of cell differentiation. AA stimulates *in vitro* differentiation of several mesenchymal cell types such as adipocytes, chondrocytes, myoblasts, osteoblasts and odontoblasts (Alcain and Buron 1994). Moreover, the differentiation of chondrocytes (Dozin *et al.* 1992), myoblasts (Mitsumoto *et al.* 1994) and osteoblasts (Torii *et al.* 1994) requires AA, presumably due to its ability to induce collagen matrix synthesis and deposition. The ability of AA to stimulate collagen secretion has been extensively studied in cultured fibroblasts. It was shown that AA increases the post-translational hydroxylation of proline (Priest and Bublitz 1967; Blanck and Peterkofsky 1975) and activates the transcription of pro-collagen genes (Phillips *et al.* 1994).

Differentiation requires the commitment of mesenchymal stem cells to a given lineage, following by induction of tissue-specific gene expression patterns. During osteoblastic differentiation AA induces expression of several osteoblastic marker proteins such as type I collagen, alkaline phosphatase (Torii *et al.* 1994), collagenase 3 (D'Alonzo *et*

al. 2002), osteocalcin (Xiao *et al.* 1997), osteonectin and tissue inhibitor of metalloproteinase 3 (Suzuki *et al.* 2003). Osteoblastic differentiation of mesenchymal cells can also be achieved when using the stable vitamin C derivative AA2P (*e.g.* Torii *et al.* 1994; Suzuki *et al.* 2003), which suggests that it is an ascorbate-dependent process, rather than a possible non-specific effect resulting from AA auto-oxidation *in vitro*. AA and AA2P can also stimulate differentiation of vascular smooth muscle cells (VSMCs) by increasing the expression of two smooth muscle-specific markers, smooth muscle-specific myosin heavy chain-1 and calponin 1 both *in vitro* and *in vivo* (Arakawa *et al.* 2003). The dedifferentiation of VSMCs is strongly implicated in the processes of atherosclerosis and restenosis after angioplasty, so the authors proposed that AA, due to its ability to maintain VSMCs in the differentiated state in the vascular wall, could have an important cardioprotective action *in vivo*.

The recent availability of human embryonic stem (ES) cells that have the ability to be differentiated *in vitro* into specialised cell types provided the opportunity to study the gene expression events related to many developmental processes. AA induces the differentiation of ES cells into cardiac myocytes and increases the expression of a number of cardiac marker genes (GATA4, α-MHC, β-MHC, ANF) (Takahashi *et al.* 2003). As noted by the authors, this effect is apparently independent from its antioxidant properties. In fact, other antioxidants are known to inhibit cardiomyocyte differentiation, while H_2O_2 enhances it (Sauer *et al.* 2000). Very recently, another group has employed cDNA microarray technology to identify AA-responsive genes in the differentiation of ES cells into neurons (Shin *et al.* 2004). In agreement with the ability of AA to enhance neuronal differentiation of ES cells, the authors reported that AA induced the expression of genes involved in neurogenesis (neuronatin, brain derived neurotrophic factor and neurotrophic tyrosine kinase receptor), neuronal maturation (double cortin and calcium/calmodulin-dependent protein kinase-like 1, growth arrest specific 7 and DNA segment human D4S114) and neurotransmission (rabphilin 3, synuclein alpha, synaptotagmin 7 and receptors for the neurotransmitters glutamate, GABA and neurotensin 3). Likewise, AA repressed the expression of genes associated with pluripotency of ES cells (developmental pluripotency associated 5 and embryonic stem cell specific gene). It was suggested that AA might be useful for the large-scale generation of neurons for future clinical treatment. In

fact, the production of dopaminergic neurons from ES cells may be an important cell source for cell replacement therapy of neurodegenerative diseases such as Parkinson's disease. In this respect, AA was also shown to increase the yield of dopaminergic neurons differentiated *in vitro* from rat embryonic central nervous system precursors (Yan *et al.* 2001). This effect could not be mimicked by any other antioxidants, suggesting a novel role for AA independent of its antioxidant properties. The analysis of gene expression changes in the neuronal differentiation following AA (200 μM) treatment was recently performed using cDNA microarray technology (Yu *et al.* 2004). In contrast with the observations of Shin *et al.* (2004), the authors reported that, despite causing a clear increase in differentiated neurons, AA did not induce changes in the expression of genes previously known to be crucial for neuronal differentiation. Instead, AA increased the expression of genes encoding for iron-binding proteins (transferrin and ferritin) and several genes that are known to be part of the cellular response to ROS, such as glutathione peroxidases, metallothioneins and glutathione-S-transferases. This suggests that the observed expression profiles may result from the pro-oxidant effect of AA and, based on that evidence, the authors hypothesised that oxidative stress may play a role in inducing neuronal differentiation.

In summary, studies with cultured cells have shown that AA can affect gene expression and this seems to be mediated by its redox effects. A schematic diagram of the proposed mechanisms of action of AA is presented in Figure 1.8. Briefly, vitamin C enters cells as ascorbate directly through sodium-dependent transporters. Alternatively, DHA generated in the extracellular space upon oxidation of ascorbate by ROS or by free metal ions is taken up through the glucose transporters and, once inside the cells, it is reduced back to AA. The intracellular AA enrichment and the oxidation events putatively generated during the AA recycling from DHA have the potential to change the cellular redox status. This and the H_2O_2 formed extracellularly during the auto-oxidation of AA can modulate the binding activity of redox-sensitive transcription factors, resulting in the activation of gene expression. The cellular effects are cell type-specific and include the activation of stress response genes, cell killing by apoptosis, cell differentiation, cell proliferation and possibly DNA repair. Some of the pro-oxidant effects described here can apparently be explained by AA auto-oxidation because they were exclusive of those vitamin C derivatives that auto-

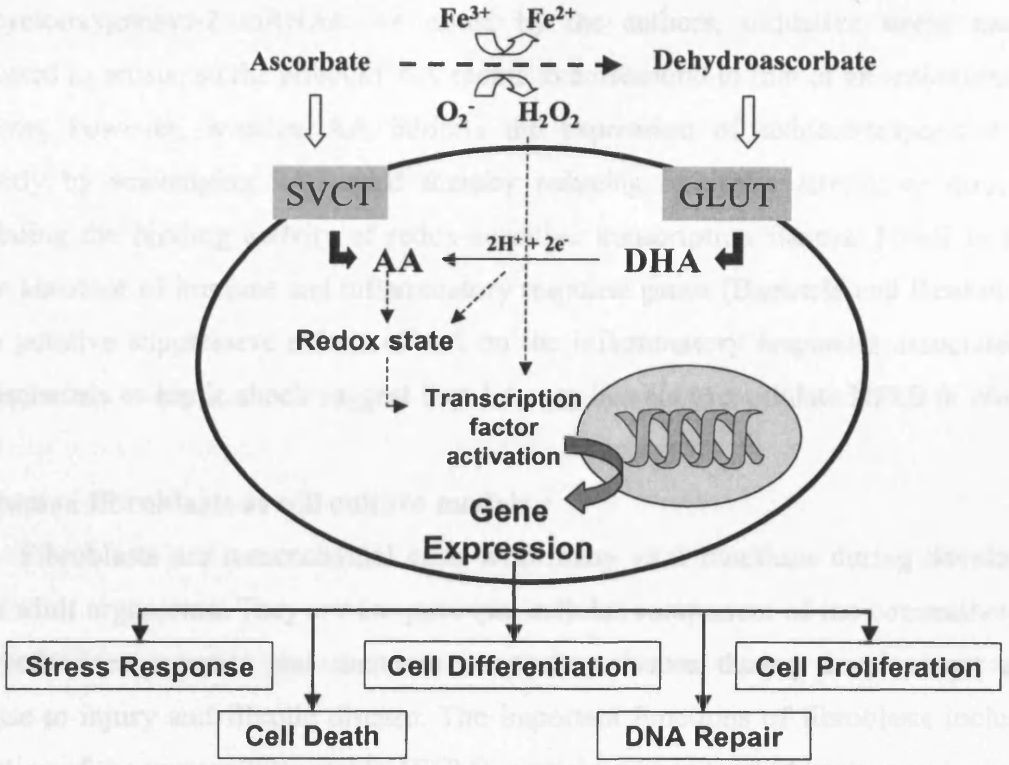


Figure 1.8. Proposed mechanisms of action of vitamin C in mammalian cells.

oxidise and/or they could be mimicked by the addition of H_2O_2 to cells, whereas other effects seemed to be more specific to vitamin C. The biological relevance of the former is still unknown, partially because the effects of AA on gene expression *in vivo* have not been thoroughly studied. In this respect, a recent report has investigated the effect of AA on gene expression of rat liver during induced septic shock (Kim and Lee 2004). Rats were subjected to polymicrobial sepsis by cecal ligation and puncture and immediately given either AA or saline intravenously. The livers were removed 24 hours later to evaluate oxidative stress and monitor expression of vasoregulatory genes that are induced in the liver in situations of oxidative stress and in inflammatory responses. Cecal ligation and puncture resulted in increased oxidative stress as demonstrated by a reduction in hepatic glutathione and a rise in lipid peroxidation. Notably, AA treatment was able to attenuate these effects. Likewise, AA inhibited or attenuated the sepsis-induced increase in the

mRNA expression levels of inducible nitric oxide synthase, HO-1, tumour necrosis factor-α and cyclooxygenase-2 mRNAs. As noted by the authors, oxidative stress has been implicated in sepsis, so the effect of AA seems to correspond to that of an antioxidant. It is unknown, however, whether AA inhibits the expression of oxidant-responsive genes indirectly by scavenging ROS (and thereby reducing oxidative stress), or directly by modulating the binding activity of redox-sensitive transcription factors. NFkB is a well-known activator of immune and inflammatory response genes (Baeuerle and Henkel 1994), so the putative suppressive effects of AA on the inflammatory responses associated with atherosclerosis or septic shock suggest that AA may be able to modulate NFkB *in vivo*.

1.5. Human fibroblasts as cell culture models

Fibroblasts are mesenchymal cells with many vital functions during development and in adult organisms. They are the principal cellular component of the connective tissue and synthesize, organise and maintain connective tissues during development and in response to injury and fibrotic disease. The important functions of fibroblasts include the deposition of the extracellular matrix (ECM), participation in wound healing and regulation of epithelial differentiation and inflammation. Fibroblasts are key players in the remodelling of the ECM. They are able to synthesise many of its constituents, such as collagens and fibronectin, and they regulate ECM turnover by synthesising ECM-degrading proteases such as matrix metalloproteases. Fibroblasts are also key elements in the process of wound healing. They invade lesions and produce ECM that serves as a scaffold for other cell types and they have cytoskeletal elements that facilitate contractions of wound healing. During development, reciprocal epithelial-mesenchymal interactions are required for the development of many organs, including the skin, eyes, lung, and other visceral organs. Finally, fibroblasts are involved in maintaining the homeostasis of adjacent epithelial tissues through the secretion of growth factors or by direct mesenchymal-epithelial interactions (Eckes *et al.* 1999).

HDFs exhibit a density-dependent inhibition of growth. They are usually found in a resting phenotype but can be activated during wound healing and fibrosis. Fibroblast activation can be induced by several stimuli produced during tissue injury. These include growth factors (*e.g.* transforming growth factor-β, epidermal growth factor, platelet-derived

growth factor and fibroblast growth factor 2) released from injured epithelial cells and infiltrating monocytes and macrophages, chemokines (*e.g.* monocyte chemotactic protein 1), ECM-degrading proteases and ROS. Fibroblast 'activation' is characterised by secretion of higher levels of ECM components and increased proliferation. Activated HDFs also secrete increased levels of ECM-degrading proteases (*e.g.* matrix metalloproteases 2, 3 and 9), growth factors (*e.g.* hepatocyte growth factor, insulin-like growth factor, epidermal growth factor, fibroblast growth factor 2, nerve growth factor), cytokines (*e.g.* interleukin-1) and chemokines (*e.g.* monocyte chemotactic protein 1). HDFs that become activated during wound healing revert to the resting phenotype once the wound is repaired. However, HDFs can remain perpetually activated in tissue fibrosis, a process that may lead to organ death. Activated HDFs are also found associated with tumours (Eckes *et al.* 1999; Kalluri and Zeisberg 2006). HDFs are implicated in the aetiology of many diseases, either directly or indirectly through the tissue fibrosis that accompanies damage to other cell types. Tissue fibrosis causes excessive deposition of ECM, eventually leading to loss of organ structure and compromising its function.

Fibroblasts are among the most easily obtainable normal mammalian cell types and they also grow well under standard cell culture conditions (Takashima 1998). For example, they can be expanded to large numbers from a small skin biopsy. Because biopsy samples can be easily obtained from skin, this tissue is the most popular source of HDFs (Takashima 1998). Skin is composed of continually renewing multilayered squamous epithelium (epidermis), connective tissue (dermis) and subcutaneous (adipose) tissue. Human epidermis contains keratinocytes (epithelial cells producing keratin intermediate filaments) and in small numbers, Langerhans cells (antigen-presenting cells of dendritic cell lineage) and melanocytes (which produce pigment granules called melanosomes). The dermis is a fibrous and filamentous connective tissue that contains fibroblasts, endothelial cells, mast cells, macrophages and occasionally other leukocyte populations. HDFs are identified by their typical spindle-shaped morphology, adherence to plastic culture vessels and the absence of specific markers for other cell lineages.

Fibroblasts grow rapidly, with a doubling time of 24-72 hr, and can be successfully propagated in culture for over 10 passages. For these reasons, fibroblasts have been widely employed in research (Takashima 1998). They have been used to study the function and

metabolism of extracellular matrix proteins and other basic aspects of cell biology, as well as genomic, biochemical and functional abnormalities in human patients and in transgenic or knockout animals. In addition, HDF cultures established from patients with inherited disorders have often been used to identify genetic abnormalities. It is worth noting that HDF lines generated from healthy human volunteers and from patients suffering from different inherited disorders are commercially available from the main tissue culture banks.

1.6. Aims of the project

AA is an important antioxidant in human plasma, where it acts as a scavenger of free radicals and protects against lipid peroxidation (Frei *et al.* 1988). However, the vitamin has other important, non-antioxidant biological functions (reviewed by Arrigoni and De Tullio 2002; Duarte and Lunec 2005), some of which relate to its ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron. AA promotes intestinal absorption of dietary non-haem Fe^{2+} iron through the divalent metal transporter 1 (see Section 1.4.5) and increases the activity of Fe^{2+}-dependent prolyl hydroxylases that participate in the synthesis of collagen and carnitine (Dejong *et al.* 1982; Schofield and Ratcliffe 2004). The ability of AA to reduce ferric to ferrous iron may, however, have deleterious consequences. In this respect, the vitamin is a well-known promoter of *in vitro* Fenton reactions. Mixtures of AA and iron (or copper) are known to induce oxidative modifications of lipids, proteins and DNA in solution (Halliwell and Gutteridge 1999). AA contributes to oxidative damage formation by recycling the metal catalyst, thus permitting another cycle of hydroxyl radical formation from H_2O_2. Although AA-mediated Fenton reactions should be controlled in the plasma and lymph of healthy individuals by efficient iron sequestration in transferrin (Winterbourn 1981, Aruoma and Halliwell 1987), redox-active, NTBI is found in the serum of individuals with iron overload, which may catalyse free radical formation in the presence of AA (Gutteridge *et al.* 1985; Hershko and Peto 1987). Additionally, cells possess a pool of labile iron that is thought to catalyse the formation of hydroxyl radicals from H_2O_2 (Greenberg and Wintrobe 1946; Halliwell and Gutteridge 1990). In fact, vitamin C supplements have been reported to enhance cell and organ damage, and eventually cause cardiac failure, in patients with iron overload (Nienhuis 1981; McLaran *et al.* 1982; Herbert

et al. 1996). It is thus possible that vitamin C may promote deleterious Fenton-type reactions in cells.

The mechanisms of AA-driven free radical generation from iron have been the subject of great discussion in the last decade. However, whilst the AA-driven Fenton reaction is well established in test tube reactions, the relevance of this pro-oxidant chemistry in cells, tissues or organisms remains unclear. The present work focused on the intracellular effects of AA under normal conditions and during oxidative stress. In particular, this study investigated whether AA modulates iron homeostasis and iron-mediated oxidative injury in human cells. Ultimately, the study aimed at determining whether AA has an antioxidant or a pro-oxidant effect inside cells. The cellular effects of AA were studied using primary normal HDFs.

It is well accepted that AA is unstable in aqueous media and, in the presence of oxygen, it oxidises to DHA. As described in Section 1.4.8, the production of ROS during extracellular AA 'auto-oxidation' may explain some contradictory findings from studies using cell culture models. The experiments presented in Chapter III were therefore aimed at better understanding the pro-oxidant effect of AA solutions in cell culture models and identifying experimental conditions that would minimise the contribution of extracellular AA auto-oxidation, thus allowing the study of intracellular effects of AA. Having established such conditions, experiments were carried out to investigate whether intracellular AA enrichment could protect or sensitise cells towards DNA damage or cell death caused by a physiologically relevant oxidant species, H_2O_2 (Chapter IV). The next series of experiments investigated the ability of AA to modulate cellular iron homeostasis by measuring changes in the intracellular LIP and in the expression of two well-established iron-regulated genes, ferritin and TFRC. HO-1 activity is also involved in cellular iron metabolism, so the effect of AA on its expression was also investigated (Chapter V). The possibility that AA regulates cellular function, through its effects on the intracellular redox state and/or on iron homeostasis, was also addressed by performing a genome-wide analysis of AA-induced gene expression in HDFs (Chapter VI).

HDFs were the cell model chosen in this study because they can be easily propagated as primary cultures for a number of passages according with standard culture conditions. HDFs, like other cell types, have a pool of intracellular free iron (Pourzand *et*

al. 1999; Zhong *et al.* 2004), which can be modulated *in vitro* by iron supplementation or chelation. In addition, HDFs express TFRC at the cell-surface (Ward *et al.* 1982; Ekblom *et al.* 1983; Wiley and Kaplan 1984; Chen *et al.* 1991) and the effects of iron supplementation or chelation on the expression of ferritin and TFRC are well-established (Ward *et al.* 1982, 1984; Knisely *et al.* 1989).

CHAPTER II

MATERIALS AND METHODS

2.1. Reagents and chemicals

Minimal essential medium (MEM) with Earle's salts and non-essential amino acids, MEM with Earle's salts containing 2mM L-glutamine, MEM amino acids, MEM non-essential amino acids, MEM vitamins solution, foetal bovine serum (FBS), Glutamax-I, Hanks' balanced salt buffer (HBSS), 10 × BlueJuice gel loading buffer and acetylated bovine serum albumin (BSA) were all purchased from Invitrogen (Paisley, UK). Sodium chloride/sodium phosphate/EDTA (SSPE) was purchased from BioWhittaker (Walkersville, Maryland, USA). Tween 20 was purchased from Pierce (Cheshire, UK). Pellet Paint and superoxide dismutase from bovine liver were purchased from Merck Biosciences (Nottingham, UK). Agarose was purchased from Helena BioSciences (Tyne & Wear, UK). Herring sperm DNA was purchased from Promega (Madison, Wisconsin, USA). Control oligo B2 and prokaryotic control cRNAs (Bio B, Bio C, Bio D, Cre) were purchased from Affymetrix (Santa Clara, California, USA). Streptavidin-phycoerythrin conjugate (SAPE) and Phen Green SK (PG SK) diacetate were purchased from Molecular Probes (Oregon, USA). DNA HyperLadder V was purchased from Bioline (London, UK). Biotinylated goat anti-streptavidin antibody was purchased from Vector Laboratories (Burlingame, California, USA). Magnesium L-ascorbate 2-phosphate was obtained from Wako Pure Chemical Industries (Neuss, Germany). Fermentas 6 × gel loading dye and Fermentas RNA ladder were purchased from Helena BioSciences (Tyne & Wear, UK). Other materials and reagents of the highest quality were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

2.2. Cell lines and culture conditions

Primary fibroblast cell lines established from skin biopsies of apparently healthy human donors were purchased from the NIGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, New Jersey, USA) at passage 3 (GM05399), 4 (GM05381), 6 (GM05659D) and 15 (GM00969D). Human breast cell lines HBL100 and MCF-7, kindly provided by Prof. Maggie Manson (Cancer Biomarkers and Prevention Group, University of Leicester, UK), were originally obtained from American Type Culture Collection (Manassas, Virginia, USA). Cells were grown as a monolayer culture in Nunclon culture flasks (Invitrogen, Paisley, UK) at 37 °C in a humidified atmosphere containing 5 % CO_2. GM969D HDFs were grown in MEM with

Earle's salts containing 2 mM L-glutamine supplemented with 2 × MEM essential amino acids, 2 × MEM non-essential amino acids, 2 × MEM vitamins and 15 % (v/v) FBS. All other cell lines were grown in MEM with Earle's salts containing non-essential amino acids supplemented with 2 mM Glutamax-I and 10 % (v/v) FBS. Cells were passaged when nearly confluent. For experiments requiring serum starvation, nearly confluent monolayers were cultured in medium with 0.5 % (v/v) FBS (low-serum medium) for 48 hours. For passaging, growth medium was removed and cells were washed in pre-warmed sterile phosphate buffered saline (PBS). Cells were incubated in PBS containing 0.05 % (w/v) porcine trypsin and 0.02 % (w/v) tetrasodium EDTA at 37 °C for 2 minutes or until cells started to separate. The flask was then gently tapped to help release the cells. Once cells were floating, growth medium was added to the flasks to inactivate the trypsin and cells were centrifuged at 300 × *g* for 4 minutes. With the supernatant removed, the tube was gently tapped to disaggregate the pellet and the cells were resuspended in an appropriate volume of growth medium and distributed into new flasks (split ratio 1:3). For applications that required seeding cells at known densities, cells were counted using a CASY Cell Counter and Analyser System (Schärfe System, Germany) or an Improved Neubauer microscopic counting chamber (Hawksley, Sussex, UK), as indicated in Section 2.7. Cells were used between passages 9-17 (GM5399), 14-17 (GM5659) or 19-21 (GM969) and then restarted from frozen stocks in order to eliminate variables related with aging. HBL100 and MCF-7 breast epithelial cells were used between passages 12-17.

HDFs were frozen at 10^6 cells/ml in cold MEM containing 50 % (v/v) FBS and 5 % (v/v) dimethylsulphoxide (DMSO). Vials containing 1 ml aliquots of the cell suspension were wrapped with paper and placed inside a polystyrene container in a - 80 °C freezer. Cells were left at - 80 °C at least overnight and transferred into liquid nitrogen storage within one week. Whenever necessary, a vial of cells was removed from the liquid nitrogen bank, placed directly into a 37 °C waterbath and shaken for up to two minutes, until fully thawed. Cells were resuspended in the vial by gently pipetting the contents up and down and transferred to a sterile tube containing about 7 ml of growth medium. Cells were centrifuged, resuspended again in an appropriate volume of fresh culture medium and added to a tissue culture flask or dish.

Cells were tested for mycoplasma contamination using a polymerase chain reaction (PCR)-based method (VenorGem Mycoplasma Detection Kit, Cambio, Cambridge, UK). Briefly, 100 µl of the cell culture medium were transferred to a sterile

tube, incubated at 99 °C for 5 minutes and briefly centrifuged to pellet cellular debris before adding to the PCR mixture. The PCR reaction was prepared by adding 27.8 µl of water, 3 µl of 50 mM $MgCl_2$, 5 µl of 10 × core PCR buffer, 10 µl of primer/nucleotide mix, 2 µl of plasmid DNA (internal control), 0.2 µl of Taq polymerase (5 U/ µl) (Stratagene, Amsterdam, Netherlands) and 2 µl of either water (no-template control), cell culture supernatant or a solution of DNA from *Mycoplasma orale* (positive control). The PCR was run in a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA) and consisted of a denaturation step at 94 °C for 10 minutes and 35 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 55 °C for 30 seconds and primer extension at 72 °C for 30 seconds. Subsequently, 5 µl of the PCR product were added to 1 µl of Fermentas 6 × gel loading dye and run on a 1.5 % agarose gel at 5 V/cm for approximately 2 cm. The gel was stained with 0.5 µg/ml of ethidium bromide (EtBr) for 15 minutes, washed with water and visualised in a UV transilluminator. Results are presented in Appendix I.

2.3. Exposure of cells to test compounds

Millimolar solutions of AA, AA2P, ferric ammonium citrate (FAC), iron chelators and H_2O_2 were prepared using PBS (pH 7.4) immediately prior to the experiments. Fresh dilutions of a 30 % stock solution of H_2O_2 in PBS were used as a source of H_2O_2. According to the information provided by the supplier, the batch of FAC used in the present work contained 17.6 % Fe by weight. Solutions were sterilised by passing through a 0.22 µm membrane and protected from direct light. The solutions were then diluted in pre-warmed medium to obtain the desired concentrations and added to the cells. Control cells received medium alone. Incubation of cells with oxidants, antioxidants, iron or iron chelators was performed as described in the text and in figure legends in the Results sections. At the end of each treatment, cells were thoroughly washed with pre-warmed, sterile PBS (pH 7.4).

2.4. Exposure of cells to ionising radiation

For DNA damage studies, nearly confluent HDFs in 12-well tissue culture plates were exposed to 15 Gy of X-ray using a Pantak industrial X-ray machine (Department of Genetics, University of Leicester) at a dose rate of 1 Gy/minute, 250 kV constant potential, 1.2 mm Cu. HDFs were irradiated on ice to prevent DNA repair. Control cells were left on ice for the same period of time.

2.5. Exposure of cells to UVA radiation

2.5.1. UVA source and dosimetry

A custom-made UV box was produced to accommodate the irradiation sources (Hybec Limited, Leicester, UK). UVA irradiation was produced by a bank of six Cleo Performance/40 watts fluorescent lamps (Philips), to which a Schott Desag M-UG2 UV transmitting absorption glass filter (HV Skan, Solihull, UK) was fitted in order to remove visible and infrared wavelengths. Spectral analysis of the lamps was performed using a single monochromator diode array spectroradiometer (Edwards and Monks 2003) with the assistance of staff from the Department of Chemistry, University of Leicester (Appendix II). UV irradiance was measured with a MP-100 UV radiometer calibrated within the range 0.1-19 mW/cm^2 and fitted with a calibrated MP-136 UVA sensor (Knight Optical Technologies, Surrey, UK). Calibration of radiometer and sensor was traceable to the National Physical Laboratory (Middlesex, UK).

2.5.2. UVA irradiation procedure

Before irradiation, cells were washed twice with sterile PBS and kept in sterile HBSS with Ca^{2+} and Mg^{2+} for irradiation. Cell irradiation was performed at room temperature through a Petri dish plastic (polystyrene) lid. The temperature at the cell layer level did not exceed 37 °C. Following UVA irradiation, cells were returned to the CO_2 incubator and incubated in growth medium at 37 °C for 4 hours.

2.6. Cell viability and proliferation assays

2.6.1. Determination of cell proliferation by cell count

For cell proliferation studies, cells were harvested by trypsinisation, seeded in tissue culture grade dishes at a low density (0.33×10^4 $cells/cm^2$) and incubated with medium containing 10 % FBS for approximately 24 hours. The culture medium was then replaced with 20, 100 or 500 μM solutions of either AA or AA2P dissolved in the same medium. Control cells were incubated with medium alone. Treatment and control media were changed daily and at the fifth day cells were harvested from triplicate wells by trypsinisation. Control cells were harvested at 0, 3, 5 and 7 days. Viable cells were counted using the CASY Cell Counter and Analyser System (Schärfe System GmbH, Germany).

2.6.2. Trypan blue exclusion assay

Cell viability was routinely assessed by the trypan blue dye exclusion assay using a haemocytometer. The haemocytometer consists of a counting chamber covered by a thick, flat coverslip at a distance of 0.1 mm above the base of the slide. The principle of the assay is that when a cell suspension is diluted with trypan blue, non-viable cells (with compromised cell membrane) become swollen, large, dark blue, whereas viable cells (with a functional cell membrane) are able to exclude the dye and hence stay small, round and colourless. An aliquot of the cell suspension was mixed with an equal volume of a 0.4 % (w/v) solution of the vital stain trypan blue by pipetting up and down. The cell suspension was pipetted slowly into an Improved Neubauer microscopic counting chamber (Hawksley, Sussex, UK) and the viable (white) and dead (blue) cells in four of the 1 mm^2 areas were counted within 5 minutes (if cells are left in trypan blue for too long even healthy cells may take up the dye). The concentration of viable cells in 1 ml of cell suspension was calculated as the number of unstained cells per 1 mm^2 area multiplied by 10,000 (the conversion of 0.1 mm^3 to 1 ml) and by the diluting factor. The percentage of viable cells was calculated as the ratio between the viable (unstained) cell count and the total (stained + unstained) cell count multiplied by 100.

2.6.3. Propidium iodide uptake assay

Cells treated in 6-well or 12-well plates were trypsinised taking care to collect the supernatant. After centrifugation at 300 × *g* for 4 minutes, cells were resuspended in 500 µl of PBS containing 5 µg/ml propidium iodide (PI) and incubated at room temperature for 5 minutes. Subsequently, 10,000 cells were acquired in a FACScan flow cytometer (Becton Dickinson, Oxford, UK) and analysed using Cell Quest software. The main cell population was gated from a forward light scatter (FSC) *versus* side scatter (SSC) scatter plot. Dead cells were identified in a FL2 *versus* FSC scatter plot by their inability to exclude PI and a decrease in cell size.

2.6.4. Colorimetric MTT assay

Cell viability/proliferation inhibition of HDFs grown on 96-well plates was determined using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann 1983). A stock solution of MTT was prepared by dissolving 5 mg MTT per ml of serum-free culture medium and filtering through a 0.22

μm filter to sterilise and remove insoluble residues. Aliquots of the stock solution were stored at -20 °C. Immediately before use, an aliquot of the MTT stock solution was thawed and diluted 1:10 in complete growth medium. Cells were washed with PBS, growth medium was replaced with 50 μl of diluted MTT solution and plates were incubated at 37 °C for up to 4 hours. The MTT solution was then removed and 100 μl of DMSO added to the wells to solubilise the coloured formazan crystals formed by the cells. Plates were gently shaken for 5 minutes in an orbital shaker at room temperature to completely solubilise the formazan and absorbance of the formazan product read at 544 nm in a FLUOstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany). The absorbance value for each sample was corrected by subtracting the mean absorbance of the reference blanks (i.e. wells containing all reagents except cells).

2.7. Cell cycle analysis by flow cytometry

Cells were harvested by trypsinisation, washed in cold PBS and fixed in 70 % ethanol at approximately 1×10^6 cells/ml while vortexing gently. Cells were kept at 4 °C for a minimum of 24 hours. Prior to analysis, cells were centrifuged at 600 × *g* for 10 minutes and resuspended in PBS with 0.1 mg/ml RNase A and 5 μg/ml PI. Following incubation at room temperature for 30 minutes, 10,000 cells were acquired in a FACScan flow cytometer (Becton Dickinson, Oxford, UK) and the data obtained were analysed with ModFitLT v2.0 software (Verity Software House, Topsham, ME, USA). Briefly, the majority of the cell population was gated in a FSC *versus* SSC plot according to the size and shape of the particles in order to exclude cell debris from the analysis. A second gate was then applied to the particles exhibiting a fluorescence peak with a certain width (FL2-W) and area (FL2-A), thus excluding aggregates from the analysis. Modfit software was then used to plot a DNA histogram as the number of cells per PI fluorescence intensity and to calculate the proportion of cells in the different phases of the cell cycle using mathematical algorithms.

2.8. Analysis of DNA damage using the alkaline comet assay

DNA damage was measured using the alkaline comet assay described by Singh *et al.* (1988) with modifications. Cells were harvested by trypsinisation and immediately placed on ice. Following centrifugation at 300 × *g* for 4 minutes at 4 °C, cells were resuspended in 0.6 % low melting point agarose at 37 °C. Quickly, eighty microliters of the agarose (containing approximately 3×10^4 cells) were dispensed onto

two glass microscope slides previously coated with 1% normal melting point agarose. The slides were kept on ice for approximately 10 minutes to allow the agarose gel to set under a glass coverslip, after which the coverslips were removed and slides left in ice-cold lysis buffer (100 mM disodium EDTA, 2.5 M NaCl, 10 mM Tris-HCl, pH 10 containing 1 % (v/v) triton X-100 added fresh) overnight. Following three washes in cold distilled water, slides were placed in ice-cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM disodium EDTA, pH ≥13) for 20 minutes and then electrophoresed at 0.7 V/cm and 300 mA for 20 minutes in a 30 × 25 cm flat-bed electrophoresis tank (Flowgen Bioscience, Nottingham, UK). Slides were washed once with neutralisation buffer (0.4M Tris-HCl, pH 7.5) for 20 minutes and washed twice with double-distilled water, then left to dry in a 37 °C incubator. All procedures were carried out under subdued light to minimise background DNA damage.

For staining, slides were re-hydrated in distilled water for 30 minutes, incubated with a freshly made solution of 2.5 μg/ml PI for 20 minutes at room temperature, washed with double-distilled water for 30 minutes and finally allowed to dry. Slides were stored in the dark until image analysis. Comets were visualised by use of an Olympus BH-2 fluorescence microscope, fitted with an excitation filter of 515-535 nm and a barrier filter of 590 nm, at a magnification of 200 ×. Images were captured by an on-line charge–coupled device (CCD) camera and analysed with the Komet Analysis software (version 5.5) from Andor Bioimaging (Belfast, UK). A total of 100 cells were analysed per sample, 50 per duplicate slide, taking care to avoid the edges of the gels as well as areas around any air bubbles. The percentage of DNA in the tail of the comet (% tail DNA) was calculated for each cell by the Komet Analysis software.

2.9. DNA microarray analysis

2.9.1. Total RNA extraction

At the end of each incubation period, cells were washed twice with PBS and lysed directly on the plate for total RNA (T-RNA) extraction using RNeasy columns (QIAGEN, Crawley, UK), according to the manufacturer's instructions. Briefly, cells were lysed by addition of 800 μl of buffer RLT and scraped with a rubber policeman. The lysate was homogenised by passing through a QI shredder column and a precipitate was formed following the addition of 1 volume of 70 % ethanol. The rest of the RNA extraction procedure was performed using the RNeasy kit according to the

manufacturer's instructions, with two minor changes: after discarding buffer RPE, columns were placed on a fresh collection tube and centrifuged for 1 minute at maximum speed to eliminate any ethanol carryover; 30 µl of RNase-free water were pipetted into the column and left for approximately 5 minutes before eluting the RNA.

T-RNA was quantified by measuring the absorbance of a 1:100 diluted solution at 260 nm in a Shimadzu UV-1201 spectrophotometer (Shimadzu Corporation, Australia). Based on the extinction coefficient of RNA in water, one A260 unit of ssRNA corresponds to 40 µg/ml. Therefore the concentration of RNA can be calculated using equation 18, where O.D.260 stands for optical density at 260 nm and d.f. stands for the dilution factor:

$$[\text{RNA}]\ (\text{ng}/\mu\text{l}) = \text{O.D.260} \times 40 \times \text{d.f.} \quad [18]$$

T-RNA integrity was investigated in the Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, California, USA), a microfluidic system for nucleic acid analysis (Mueller *et al.* 2000), using the Eukaryote Total RNA Nano assay according to the manufacturer's specifications. T-RNA samples were diluted 1:10 in RNase-free water before loading on the chip. T-RNA was frozen at -70 °C until further analysis.

2.9.2. Double-stranded cDNA synthesis

The SuperScript kit (Invitrogen, Paisley, UK) was used to convert the mRNA in the T-RNA samples into double-stranded cDNA (ds-cDNA) by reverse transcription with an oligo-dT primer. Initially, first strand cDNA was formed in a reaction containing 16 µg of T-RNA, 100 pmol of T7-$(dT)_{24}$ oligomer (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$ -3'), 10 mM dithiothreitol (DTT), 500 µM each deoxynucleotide triphosphate (dNTP) and first strand buffer (50 mM TrisHCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$). This mixture was incubated at 70 °C for 10 minutes and placed on ice for 5 minutes. Following this incubation, Superscript II reverse transcriptase (400 U) was added and incubated for one hour at 42 °C.

The second strand cDNA synthesis was performed by adding to the solution containing the first strand cDNA, 200 µM each dNTP, 10 U of *E. coli* DNA ligase, 40 U of *E. coli* DNA polymerase I, 2 U of *E. coli* RNase H and second strand buffer (20 mM TrisHCl, pH 6.9, 90 mM KCl, 4.6 $MgCl_2$, 0.15 mM β–NAD^+, 10 mM $[NH_4]\ SO_4$). The reaction was incubated for 2 hours at 16 °C. Following this incubation, 10 U of T4

DNA polymerase were added and incubated for 5 minutes at 16 °C. The reaction was stopped by the addition of 27.4 mM EDTA. The resulting ds-cDNA was extracted once against 1:1 phenol-chloroform and once against chloroform and removed from the aqueous phase by the addition of 0.1 volumes of 3 M sodium acetate and 2 volumes of ethanol, using Pellet Paint as a carrier. Pellet was washed in ethanol, dried and resuspended in an appropriate volume of RNase/DNase-free water.

2.9.3. *In vitro* transcription

Synthesis of labelled complementary RNA (cRNA) was performed by *in vitro* transcription using the BioArray HighYield RNA Transcript Labelling kit (ENZO, Farmingdale, USA). Briefly, the ds-cDNA synthesised was used as template and T7 RNA polymerase was added to perform *in vitro* transcription (IVT) from the T7 RNA polymerase promoters in the template. Biotin-labelled ribonucleotides (Bio-CTP, Bio-UTP) were included in the reaction along with non-labelled ribonucleotides to produce biotin-labelled cRNA molecules. The reaction was incubated at 37 °C for 5 hours. A 15-minute incubation with 2 U of RNase-free DNase I (Roche, Mannheim, Germany) was performed to digest the template cDNA.

Quantification of cRNA was performed in a spectrophotometer by measuring the absorbance at 260 nm and the yield calculated after subtracting the carry-over of unlabelled T-RNA according to equation 19, where RNA_m is the amount of cRNA measured after the IVT reaction, $T\text{-}RNA_i$ is the starting amount of T-RNA and y is the fraction of cDNA used in the IVT reaction:

$$\text{adjusted cRNA yield} = RNA_m - (T\text{-}RNA_i)(y) \qquad [19]$$

Size distribution of the labelled transcripts was estimated by running an aliquot of each sample on a formaldehyde denaturing 1.2 % agarose gel prepared in 1 × MOPS (4-Morpholine propanesulfonic acid) buffer (20 mM MOPS, 5 mM Sodium acetate, 1 mM EDTA, pH 7.0). The gel was pre-equilibrated in 1 × running buffer (1 × MOPS buffer with 0.74 % formaldehyde) for at least 30 minutes before the electrophoresis. The cRNA (1.5 µg) was added to 1.2 µl 10 × MOPS buffer, 1.7 µl formaldehyde, 5 µl formamide and diethyl pyrocarbonate (DEPC)-treated H_2O up to 10 µl and heated to 65 °C for 5 minutes. Following the denaturation step, 2 µl of Fermentas 6 × gel loading dye were added to the samples or the Fermentas RNA ladder, and these were loaded on the gel. Electrophoresis was performed in a flat-bed gel tank fitted with a Pharmacia Biotech Electrophoresis Power Supply-301 at 5 V/cm for approximately 40 minutes.

The gel was then stained in 0.5 μg/ml EtBr for 15 minutes and subsequently destained in DEPC-treated water for 1 hour. RNA was visualised in a UV transilluminator using the Chemi Genius^2 Bioimaging System in conjunction with GeneSnap software (Syngene, Cambridge, UK).

2.9.4. cRNA hybridisation to probe arrays

Complementary RNA (20 μg) was fragmented in a buffer containing 40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate for 35 minutes at 94 °C. The efficiency of the fragmentation was checked by denaturing gel electrophoresis as described above. Fragmented cRNA was added to the hybridisation buffer (100 mM 2-morpholinoethanesulfonic acid, MES, 1 M [Na^+], 20 mM EDTA, 0.01 % Tween 20) with 0.1 mg/ml herring sperm DNA and 0.5 mg/ml acetylated BSA. The hybridisation buffer was spiked with 50 pM control oligonucleotide B2 and the prokaryotic cRNA controls Bio B, Bio C, Bio D and Cre at 1.5, 5, 25 and 100 pM, respectively. The hybridisation mixture was heated to 99 °C for 5 minutes, cooled to 45 °C for 5 minutes and centrifuged at 14,000 × *g* for 5 minutes. A small volume of the hybridisation mixture (80 μl) containing 5 μg of labelled cRNA was hybridised to a GeneChip Test 3 Array (Affymetrix, Santa Clara, California, USA) to assess target quality and hybridisation efficiency prior to utilising a genome array. The hybridisation solution was added to the pre-wet arrays and allowed to hybridise overnight at 45 °C with permanent rotation at 60 rpm in a Hybridisation Oven 640 (Affymetrix, Santa Clara, California, USA). With the quality control criteria in the test array satisfied, the hybridisation mixture containing 15 μg of labelled cRNA was hybridised to a GeneChip Human Genome U95Av2 array (Affymetrix, Santa Clara, California, USA) following the same procedure.

2.9.5. Washing and staining probe arrays

Probe array washing and staining were performed according to specific Gene Chip protocols (Affymetrix, Santa Clara, California, USA) in the Fluidics Station 400 (Affymetrix, Santa Clara, California, USA). Briefly, Test 3 or U95A arrays were washed in a non-stringent wash buffer containing 6× SSPE, 0.01 % Tween 20, 0.005 % antifoam at 25 °C, then with a stringent buffer containing 100 mM MES, 0.1 M [Na^+], 0.01 % Tween 20 at 50 °C. Following this wash, probe arrays were stained with a

solution containing 10 μg/ml of SAPE conjugate dissolved in a buffer containing 100 mM MES, 1 M [Na^+], 0.05 % Tween 20, 0.005 % antifoam and 2 mg/ml acetylated BSA. After staining, the probe arrays were exposed to 3 μg/ml goat biotinylated anti-streptavidin antibody in a buffer as above containing 0.1 mg/ml normal goat IgG. Finally, the probe arrays were re-stained with SAPE as indicated above.

2.9.6. Scanning and analysing probe arrays

Probe arrays were scanned twice at 570 nm using a GeneArray laser scanner (Agilent, Palo Alto, California, USA) and the fluorescent intensity of the scanned image registered in CEL intensity files. GeneChip Analysis Suite software version 4.0 (Affymetrix, Santa Clara, California, USA) was used to analyse the scanned image and retrieve quality control information including noise and background, scaling factor, presence of spiked hybridization controls and the number of genes present per array.

2.9.7. Data analysis

DNA-Chip Analyser (dChip) software (Li and Wong 2001) was used to automatically select probes, detect outliers/artefacts and calculate model-based expression values from CEL intensity files. The software was used to analyse arrays individually for presence/absence and hybridisation intensity of each probe set and to normalise the fluorescence intensity over multiple arrays. dChip was also used to identify genes that were differentially expressed between two samples (comparative analysis). The genes that satisfied the filtering criteria were those determined to be present in at least one of the samples being compared, with a fluorescence intensity value higher than 50, whose expression levels increased/decreased relative to control in both independent experiments and for which the averaged fold change of expression was ≥ 1.9 or ≤ -1.9. Finally, dChip was employed to determine if the number of genes in an expression cluster (up- and down-regulated genes) with a given biological function was greater than the number expected by random chance. The P values for observing the frequencies of genes in particular functional categories were calculated as described by Cho *et al.* (2001). To avoid false positives, only the most significantly enriched functional categories ($P < 0.000001$) were reported.

2.10. Real-time reverse transcription PCR

2.10.1. Total RNA extraction

T-RNA was extracted using a modification of the guanidine thiocyanate and phenol-based method of Chomczynski and Sacchi (1987). All reagents used were of molecular biology grade and purchased from Sigma-Aldrich (Poole, UK). Briefly, cells were lysed directly on the culture dish using 1 ml of TRI Reagent, homogenised by repeated pipetting and allowed to stand for 5 minutes at room temperature. At this stage, samples may be frozen at -80 °C for up to one month until RNA extraction. Samples were fully defrosted immediately prior to resuming the extractions. After adding 200 µl of chloroform, samples were vigorously shaken and centrifuged at 12,000 × *g* for 15 minutes at 4 °C. The aqueous phase was transferred to a fresh tube and RNA was precipitated by adding 500 µl of isopropanol and allowing samples to stand for 10 minutes at room temperature. The RNA precipitate was pelleted by centrifugation 12,000 × *g* for 10 minutes at 4 °C and washed with 1 ml of 75 % ethanol. At this stage, samples may be stored at -20 °C for up to several months until ready to finish the extractions. Samples were then vortexed gently and centrifuged at 7,500 × *g* for 5 minutes at 4 °C. Supernatants were removed and the RNA pellets were air-dried for approximately 10 minutes at room temperature and then dissolved in RNase-free water at 55 °C and further incubated at 55-60 °C for 10 minutes to facilitate dissolution.

T-RNA quantification was performed by measuring the absorbance of a 1:100 diluted solution at 260 nm in a GeneQuant II RNA/DNA calculator spectrophotometer (Pharmacia Biotech, Cambridge, UK), as described in Section 2.9.1. T-RNA integrity was assessed by running an aliquot of each sample on formaldehyde denaturing 1.2 % agarose gel as described in Section 2.9.3.

2.10.2. DNase digestion and first-strand cDNA synthesis

Contaminant genomic DNA was digested using Turbo DNA-free kit (Ambion, Huntingdon, UK) according to the instructions supplied by the manufacturer. Briefly, 10 µg T-RNA were adjusted to a volume of 20 µl with RNase-free water and added to 2 µl of 10× Turbo DNase buffer and 1 µl of Turbo DNase (2 U/µl). Samples were gently mixed and incubated in a water bath at 37 °C for 30 minutes. Reactions were stopped by adding 2 µl of the DNase inactivation reagent and incubating at room temperature for 2 minutes, mixing occasionally. After centrifugation at 10,000 × *g* for 1.5 minutes, supernatants were transferred to a fresh tube and stored at -80 °C.

The first-strand cDNA synthesis was generated using the StrataScript first-strand synthesis system (Stratagene, Amsterdam, Netherlands) according to the manufacturer's

instructions. Briefly, 5 μg of DNased-RNA were added to 2 μl of 10× first strand buffer, 1 μl of oligo(dT) primer (0.5 μg/ μl), 0.8 μl dNTP mix (25 mM each dNTP) and RNase-free water to a volume of 19 μl. The mixture was incubated at 65 °C for 5 minutes to denature the RNA and then cooled down at room temperature for 10 minutes to allow primers to anneal. Then 0.5 μl of RNase Block (40 U/μl) and 1 μl of Strata Script reverse transcriptase (50 U/μl) were added, reactions mixed gently and incubated in a temperature controlled thermal block (Peltier Thermal Cycler PTC-200, MJ Research, Hemel Hempstead, UK) at 42 °C for 1 hour. The reaction was terminated at 70 °C for 15 minutes and samples stored at -20 °C until subsequent use for PCR amplification.

2.10.3. Primer design

Primer sequences for the real-time reverse transcription (RT)-PCR amplification of specific transcribed genes were designed using the Primer 3 software freely available on the WWW at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi (Rozen and Skaletsky 2000). Primers were designed to generate an amplicon size ranging between 80-105 bp, as short amplicons are thought to amplify more efficiently (Bustin 2000). Optimal primer length was 20-25 bases, G/C content was between 40-60 % and their melting temperature (Tm) did not differ by more than 2 °C, ranging between 57-63 °C. Primer specificity was tested against the human genome and expressed sequence tag (EST) databases using the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). The folding of the amplification product was tested using DNA *mfold* web server on the WWW at http://www.bioinfo.rpi.edu/applications/mfold/old/dna/ (Zuker 2003) in order to avoid primers whose resulting amplicons form secondary structures affecting the PCR reaction. Finally, primer sequences were tested for the formation of dimers, hairpin loops and primers duplexes that could also affect the PCR reaction using Vector NTI suite (Invitrogen, Paisley, UK). Primers were designed for nine genes: hypoxanthine phosphoribosyltransferase 1 (HPRT1), transferrin receptor (TFRC), haem oxygenase-1 (HO-1), cyclin B1 (CCNB1), DNA topoisomerase II α (TOP2A), tyrosyl-tRNA synthetase (YARS), low-density lipoprotein receptor (LDLR), 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR) and acetyl-Coenzyme A acetyltransferase 2 (ACAT2). Primer sequences for cyclin-dependent kinase inhibitor

p21 and growth arrest and DNA damage-inducible 45 α (GADD45α) were kindly provided by Dr. G. Almeida (Department Cancer Studies and Molecular Medicine, University of Leicester). The Forward and Reverse oligonucleotide sequences and the expected product length are listed in Table 2.1.

2.10.4. Amplification conditions

Real-time RT-PCR was performed on the MX4000 spectrofluorometric thermal cycler (Stratagene, Amsterdam, Netherlands) using the SYBR Green Jumpstart™ *Taq* ReadyMix without $MgCl_2$ (Sigma-Aldrich, Poole, UK) according to the manufacturer. The $MgCl_2$ and primer concentrations were optimised in order to maximise amplification (*i.e.* lower Ct values) and minimise primer dimer formation and non-specific amplification. $MgCl_2$ concentrations ranged between 2 and 3.5 mM, and primer concentrations ranged between 150 and 200 nM. A volume of 1 µl of cDNA solution (250 ng of total RNA) was used for the amplification of the specific cDNA targets and the PCR was carried out as follows: initial denaturation at 95 °C for 4 minutes, followed by 40 cycles of 95 °C for 30 seconds, 55 °C or 57 °C for 1 minute and 72 °C for 30 seconds. After these cycles, a dissociation curve was generated by gradually increasing the temperature 1 °C at a time (from 55 to 95 °C) and by measuring the fluorescence at the end of each temperature increase. SYBR Green fluorescence was collected using the FAM filter set (excitation wavelength 492 nm; emission wavelength 516 nm) and normalised to the signal of the reference dye, ROX (excitation wavelength 585 nm; emission wavelength 610 nm), to correct for differences caused by sample volume or dye concentration. All reactions were run in duplicate. No-template controls were always included.

2.10.5. Relative quantification and product specificity

Threshold cycle (Ct) values were determined by the Mx4000 software using an automatically set fluorescence threshold. Standard amplification curves were generated for each target by amplifying serial dilutions of a standard cDNA containing the transcribed gene of interest (dilution series was 1, 1:10, 1:100 and 1:1000 corresponding to 250, 25, 2.5 and 0.25 ng of total RNA input, respectively) to verify that there was a linear relationship between the Ct and the log (RNA input) (i.e. the slope of the standard curve was between –3.5 and –3.2).

Table 2.1. Primer sequences and respective expected product length.

Gene	Length (bp)	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
HPRT1	103	GCAGACTTTGCTTTCCTTGGTCAG	GTCTGGCTTATATCCAACACTTCGTG
TFRC	84	TCTGACACGTCTGCCTACCCATTCG	TATGATGGTTCACTCACGGAGCTTCG
HO-1	102	CAGCAACAAAGTGCAAGATTCTGC	AGTGTAAGGACCCATCGGAGAAGC
GADD45α	85	AGGATTACAGAAACTGATGCCAAGGG	TCCATCTGCAAAGTCATCTATCTCCG
p21	91	CCCAAACACCTTCCAGCTCCTGT	GTCTAGGTGGAGAAACGGGAACCAG
ACAT2	97	TGGGCTTACACCTTTAGCACGGATAG	AGCTTGCTTTATGGCTGGAATTGGT
CCNB1	104	ATGATGTGGATGCAGAAGATGGA	TCTGACTGCTTGCTCTTCCTCAA
HMGCR	96	GCCTGTTTGCAGATGCTAGGTGTTC	CATTACGGTCCCACACACAATTCG
LDLR	85	GCAAGTGGCTTTCAACACACAACAG	GGGTTTGGCTTAGAGATTGGTGGATG
TOP2A	94	TCAAGCCCTCCTGCTACACATTTC	TTTGCTGCTGTCTTCTTCACTGTCA
Tyrosyl tRNA synthetase	104	GCTATTCAAAACGGGTCCATCT	CCGATCAAGGAGATCAATCTTGG

The quantity of each target gene was estimated from the respective standard curve and normalised against the quantity of the endogenous control gene (HPRT1). The final gene expression value in each treated sample was subsequently determined as a ratio of the gene expression in the calibrator (control) sample.

The amplification of a single product was verified by the existence of a single peak in the dissociation curve and further assessed by gel electrophoresis by running a 10 µl aliquot of the PCR product and 2 µl of 6 × loading dye along with 5 µl of DNA HyperLadder V on a 3 % agarose gel. Electrophoresis was run at 60 V for approximately 1 hour and the gel stained with 0.5 µg/ml EtBr for 15 minutes, washed with distilled water and visualised under UV.

2.11. Measurement of intracellular LIP

Relative changes in the intracellular LIP were measured using an adaptation of the method of Petrat *et al.* (1999). The method is based on quenching the fluorescence of the transition metal indicator PG SK by intracellular iron and its subsequent dequenching upon addition of a strong membrane-permeable iron chelator. After the appropriate treatment in 96-well plates, cells were washed with pre-warmed (37 °C) PBS and loaded with 10 µM PG SK diacetate in Earle's MEM without FBS and without phenol-red for 10 minutes at 37 °C. Cells were then washed three times with pre-warmed (37 °C) MEM. Fluorescence of the metal indicator PG SK (excitation maximum 485 nm, emission maximum 520 nm) was measured before and 15 minutes

after the addition of the highly permeable chelator 2,2'- bipyridyl (BIP) (5 mM) in a FLUOstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany) and expressed as arbitrary fluorescence units (AFU). The increase in fluorescence produced by the chelator (Δ AFU) is proportional to the size of the intracellular LIP. The size of the intracellular LIP under different treatments was compared using equation 20, where F1 and F2 stand for fluorescence before and after the addition of the chelator, respectively, as described by Darbari *et al.* (2003):

$$\text{Fractional increase in fluorescence } (\Delta F) = (F2 - F1)/F2 \qquad [20]$$

2.12. Measurement of extracellular and intracellular AA

For the detection of extracellular AA in cultures of GM5399 HDFs and serum starved GM969 HDFs, a 0.5 ml medium aliquot was collected at each time point following AA addition and added to 4.5 ml of ice cold 10 % metaphosphoric acid (MPA). The solution was centrifuged at 1260 × *g* for 10 minutes at 4 °C to remove any debris and the supernatant kept at -70 °C until ready to analyse.

For the detection of intracellular AA in serum starved GM969 HDFs, cells were harvested by trypsinisation at the same time points, centrifuged at 4 °C and resuspended in cold PBS. At this stage an aliquot of the cell suspension was used for cell count using the CASY Cell Counter and Analyser System. Cells were centrifuged again at 4 °C and the cell pellet resuspended in 250 μl of ice-cold 10 % MPA, mixed by gentle vortexing and kept at -70 °C until ready to analyse. For analysis, samples were defrosted on ice, sonicated on ice for 5 minutes, centrifuged at 14,000 × *g* for 3 minutes at 4 °C and the supernatant collected. From this step onwards, samples were processed in the same way as extracellular AA samples.

AA was measured by high performance liquid chromatography (HPLC) with UV detection. The HPLC included an isocratic LC pump 250 (Perkin Elmer, Norwalk, CT, USA), an auto-sampler model 542 (ESA) and a UV detector UVD340S (Dionex, Camberley, UK). AA was separated on a Luna 5u C18 (2) HPLC column (150 × 4.6 mm) (Phenomenex, Macclesfield, UK) with an injection volume of 50 μl. The mobile phase consisted of 15 mM phosphate buffer. The flow rate was 0.4 ml per minute. The detector was set at a wavelength of 254 nm. The retention time of AA was approximately 5 minutes. AA was quantified using a standard curve. The standards corresponded to AA dissolved in MPA at 5, 10, 20, 30 and 50 μM. Intracellular AA concentration was normalized for cell number and expressed as fmol/cell.

For the detection of intracellular AA in GM5659 HDFs, cells were incubated in 6-well plates and AA concentration was determined by HPLC, according to the method of Rose and Bode (1995). At each time point, culture medium was removed and intracellular AA was extracted by treating cells with 5 % (w/v) trichloroacetic acid for 20 minutes, at 4 °C. 20-µl aliquots were injected in a C-18 reverse-phase column (Resolve; Waters, USA). The mobile phase (0.2 M KH_2PO_4/H_3PO_4, pH 3.0) was delivered at a flow rate of 1 ml/minute, and AA was detected by an electrochemical detector (ESA Coulochem III) with a Model 5011 analytical cell. Determinations were performed at potentials of –0.35 and 0.2 V applied on upstream and downstream electrode, respectively. Calibration was based on external standards using stock solutions (100 µM) of AA prepared in 50 mM phosphoric acid and stored at -20 °C. Working solutions were prepared daily in mobile phase. Intracellular AA concentration was normalised for total protein and expressed as nmol/mg protein.

2.13. Detection of cell-surface expression of TFRC

HDFs were harvested from a 60 mm diameter Petri dish by brief incubation in an enzyme-free dissociation solution (Invitrogen, Paisley, UK), centrifuged and resuspended in 100 µl of diluent (PBS containing 1 % (w/v) BSA and 0.1 % (w/v) NaN_3), then incubated with 5 µl of mouse monoclonal anti-human CD71 (Sigma-Aldrich, Poole, UK) or an equal concentration of isotype-matched, non-specific mouse immunoglubulin (mouse IgG1κ) (Sigma-Aldrich, Poole, UK) at room temperature for 30 minutes. Cells were washed three times in 2 ml of diluent by centrifugation at 500 × *g* for 5 minutes and then incubated with 100 µl of a 1:200 dilution of fluorescein-5-isothiocyanate (FITC)-conjugated anti-mouse IgG goat polyclonal antibody (Abcam, Cambridge, UK) at room temperature in the dark for 30 minutes. After three washes, cells were resuspended in 500 µl of diluent and kept on ice until analysed in a FACScan flow cytometer (Becton Dickinson, Oxford, UK). Analysis was performed with Cell Quest software (Becton Dickinson, Oxford, UK). Briefly, the main cell population was gated from a FSC *versus* SSC scatter plot. A histogram was plotted as the number of cells per FL1 fluorescence intensity. Levels of cell-surface TFRC were expressed as a percentage of the fluorescence in control cells within the same experiment.

2.14. Analysis of cellular ferritin

HDFs treated in 6-well plates were washed three times with ice-cold PBS and scraped from the plates in 70 µl of ice-cold complete lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0 to which 1 mM phenylmethanesulfonyl fluoride and 1 % Igepal were added immediately prior to use). Cell lysates were centrifuged at 7500 × *g* for 10 minutes at 4 °C and supernatants were collected and stored at -80 °C until ready for analysis. Analysis of cellular ferritin content was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) (BioCheck, Inc., Burlingame, CA, USA). The assay was performed in microtiter wells coated with a rabbit anti-ferritin antibody. The test samples or standards (20 µl) were allowed to react simultaneously with the solid phase antibodies and with mouse monoclonal anti-ferritin conjugated to horseradish peroxidase (100 µl), resulting in ferritin molecules being sandwiched between both types of antibodies. Following a 45-minute incubation at room temperature, wells were washed 5 times to remove unbound labelled antibodies. A solution of 3,3',5,5'-tetramethylbenzidine (100 µl) was added and incubated at room temperature for 20 minutes, resulting in the development of a blue colour. The colour development was terminated by adding 100 µl of 1N HCl and the resulting yellow colour was measured spectrophotometrically by reading the absorbance at 450 nm in a FLUOstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany) within 15 minutes. A standard curve was constructed by plotting the mean absorbance of each reference standard against its concentration in ng/ml (0, 15, 80, 250 or 500 ng/ml). The concentration of ferritin in each sample was calculated from the standard curve using the mean absorbance value. According to the supplier, the assay sensitivity is 5.0 ng/ml. Levels of ferritin were expressed as ng ferritin per mg total protein.

2.15. Protein quantification

Protein concentration was estimated according to the method of Bradford (1976) using the Bio-Rad protein assay. Briefly, 10 µl of sample were added to 1 ml of diluted Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hemel Hempstead, UK), samples were vortexed and incubated at room temperature for 5 minutes, before measuring the absorbance at 595 nm in a U-3010 spectrophotometer (Hitachi, Wokingham, UK). Protein concentrations were estimated from a standard curve prepared by diluting a BSA standard (Bio-Rad Laboratories, Hemel Hempstead, UK) to a final concentration of 0-10 µg/ml.

2.16. Statistical analysis

Unless otherwise stated, results are expressed as mean values ± S.D. for at least 3 independent samples. Statistical evaluation was performed using Minitab software (Minitab Ltd, Coventry, UK). Significance of the differences in mean values among multiple groups was estimated by two-tailed one-way analysis of variance (ANOVA) with *post-hoc* Tukey's test. When sample distribution was clearly not normal, significance of the differences in mean values among samples was determined by non-parametric Mann Whitney test. Levels of cell-surface TFRC were expressed as a ratio of the matched control and differences were compared by a two-tailed one-sample t-test. The relationship between two variables was assessed by simple linear regression analysis. Unless otherwise stated, statistical significance was assumed at $P < 0.05$.

CHAPTER III

TRANSIENT PRO-OXIDANT EFFECT OF VITAMIN C IN CELL CULTURE MODELS: THE ROLE OF H_2O_2 AND TRANSITION METALS

3.1. Introductory notes on the alkaline comet assay

In the last decade, the single-cell gel electrophoresis assay (or comet assay) has become a standard method for assessing DNA damage, with applications in genotoxicity testing, human and environmental biomonitoring and fundamental studies on DNA damage and repair (reviewed by Rojas *et al.* 1999; Kassie *et al.* 2000; Collins 2004). It is a simple, rapid and sensitive microelectrophoretic technique for the direct visualisation of several types of DNA damage in single cells. The first description of the comet assay was by Ostling and Johanson (1984), who performed the assay under neutral pH. Subsequently, Singh *et al.* (1988) greatly increased the sensitivity of the method by performing it under alkaline conditions. The general procedure for DNA damage detection by the alkaline comet assay consists of preparing a single cell suspension (*e.g.* from blood, cells in culture or disaggregated tissue) embedded in low melting point agarose (LMP) and placing this mixture as a thin gel on a pre-coated microscope slide. The cells are then lysed with detergent and high salt concentration to remove plasma and nuclear membranes and disrupt the nucleosomes, leaving nucleoid bodies composed of supercoiled loops of DNA attached to some of the scaffolding proteins that are part of the nuclear matrix. Prior to electrophoresis, DNA is allowed to unwind by immersing the slides in alkaline electrophoresis running buffer, which contains low salt, no detergents and pH > 13. The denatured nucleoid bodies are then subjected to limited electrophoresis (*i.e.* short duration and low voltage). The damaged, relaxed DNA is able to migrate away from undamaged DNA during electrophoresis, leading to the formation of what appears a 'comet', in which the 'head' consists of the nucleoid body containing intact DNA and the 'tail' consists of the damaged DNA (Figure 3.1). The amount of DNA in the tail is thus proportional to the level of damaged DNA in the respective cell. Following electrophoresis, samples are neutralised and the DNA on the gels is stained with a fluorescent dye (more commonly PI, EtBr or DAPI) to allow comet visualisation and analysis using a fluorescence microscope. DNA damage can be estimated by visually scoring the slides according to the appearance of the comets. Typically, 5 classes of damage are considered ranging from 0 (intact nucleoid with nearly all the DNA in the 'head') to 4 (highly damaged nucleoid with nearly all the DNA migrated to the 'tail'). A final score is obtained for each sample as the sum of all individual scores (Collins 2004). Alternatively, a series of image analysis software packages have been designed to determine DNA damage parameters of individual cells in a sample population. The most commonly used parameters are the

percentage of migrated DNA (or % tail DNA), tail length and tail moment. Tail length is susceptible to be affected by changes in the threshold settings used for image analysis and hence it becomes difficult to compare results inter-laboratories. Tail moment is a parameter that takes into account tail length and tail intensity, and consequently it suffers from the same limitations. Olive *et al.* (1990) defined Olive tail moment as the product of the percentage DNA in the comet tail and the distance between the centres of gravity for DNA in the head and tail. The percentage of DNA in tail (% tail DNA) is linearly related to strand break frequency over a wide range of damage levels, is independent from threshold settings and gives a clear indication of the appearance of the comets, so the results are easily interpreted by researchers in different laboratories. A potential drawback of using % tail DNA as a measure of DNA damage is the fact that it assumes that the staining efficiency of the fluorescent dye is the same for migrated (*i.e.* damaged) and non-migrated (*i.e.* intact) DNA. However, commonly used fluorescent intercalating dyes, such as EtBr or PI, bind more efficiently to double-stranded DNA than to single-stranded DNA. DNA is denatured during the alkaline treatment and, even though the supercoiled intact loops of DNA in the head of the comet renature upon neutralisation, the tail of the comet is thought to contain a high proportion of single-stranded DNA (discussed by Collins 2004).

The conditions of the lysis and the pH of the unwinding and electrophoresis buffer determine the sensitivity, the useful dynamic range and to some extent even the type of DNA lesions revealed by the assay (Collins 2004). Whilst the alkaline conditions (pH > 13) employed by Singh *et al.* (1988) allow for the detection of DNA double-strand breaks (DSB) and single-strand breaks (SSB), alkali labile sites and incomplete excision repair sites, Olive *et al.* (1991) have devised a neutral (pH 7-8) version of the assay that can measure DSB.

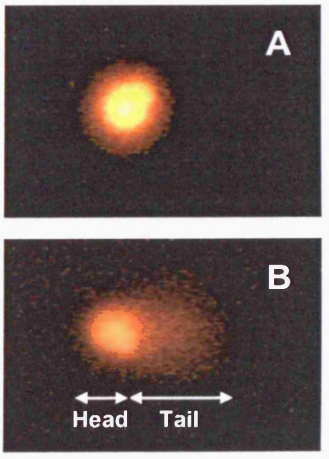


Figure 3.1. Typical appearance of agarose-embedded human fibroblasts processed in the alkaline comet assay and stained with propidium iodide (original magnification × 200). (A) Appearance of a control cell, yielding an intact nucleoid body where most of the DNA is located in the head of the comet. (B) Appearance of a cell exposed to a genotoxic agent (H_2O_2) before being processed in the comet assay. The existence of a tail reflects the DNA damage.

3.2. Specific aims of study

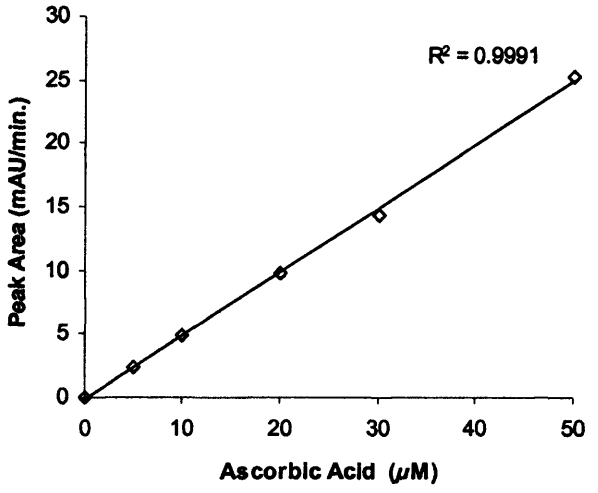
AA is unstable in aqueous media and in the presence of oxygen it oxidises to DHA, generating AFR and H_2O_2. It has been proposed that the cytotoxic effect of AA to cells in culture is due to the H_2O_2 formed from its auto-oxidation and that the latter only occurs in the presence of trace amounts of metals in the cell medium. The specific aim of this work was to study the pro-oxidant effects of vitamin C solutions in cell culture models by investigating the involvement of extracellular H_2O_2 and metals. In particular, this work aimed at understanding how AA auto-oxidation induces DNA damage and affects cell growth/viability endpoints. This should help to define conditions by which the effects of intracellular AA accumulation can be studied without the interference of extracellular AA auto-oxidation. In addition, these experiments should help address the contradictory findings of studies of the effects of vitamin C on cell culture models and clarify the possible physiological relevance of the often-cited pro-oxidant effect of vitamin C solutions.

3.3. Results

3.3.1. Vitamin C stability in cell culture medium

The concentration of AA in cell culture medium was determined by HPLC with UV detection, as described in Materials and Methods. A representative calibration curve is shown in Figure 3.2A. To investigate the stability of AA in cell culture media, HDFs were incubated with 100 μM AA in either low-serum medium (0.5 % FBS) or complete growth medium (10 % FBS) and the AA concentration in the medium was determined at 0, 2, 6, 12 or 24 hours of incubation (Figure 3.2B). Approximately 40 % of the AA was lost from culture media within 2 hours of incubation and only about 20 % of the initial AA concentration was present at 12 hours of incubation. Finally, more than 90 % of the AA disappeared from the culture media within 24 hours. It is worth noting that AA disappeared from culture medium regardless of the presence of serum. These data are in agreement with the observations of other authors (Peterkofsky 1972; Franceschi *et al.* 1995; Chepda *et al.* 2001a).

A



B

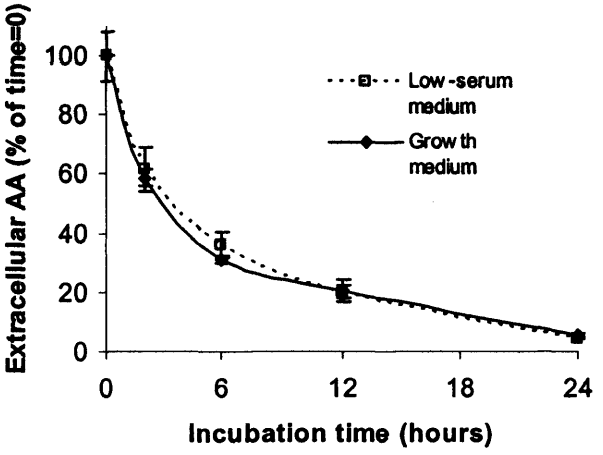


Figure 3.2. Measurement of AA concentration in cell culture medium. AA concentration was determined by HPLC with a UV detector as described in Materials and Methods. A, Example of a calibration curve generated using external AA standards. B, HDFs were incubated with 100 μM AA in low-serum medium (0.5 % FBS) or complete growth medium (10 % FBS) and the AA concentration in the medium was determined at 0, 2, 6, 12 or 24 hours of incubation. Results are the mean ± S.D. from 3 separate determinations.

3.3.2. Effects of vitamin C on cell viability and proliferation

Cell viability was determined using the MTT assay. Preliminary experiments were undertaken to confirm the linearity between the number of cells and the production of formazan. HDFs were seeded at different densities (between 0.375×10^3 and 6×10^3 cells per well) in flat-bottomed 96 well plates and left to adhere to bottom of plates for 24 hours at 37 °C. The MTT assay was performed in the next day as indicated in

Materials and Methods (Section 2.6.4). A linear relationship was obtained between cell number and the absorbance of the formazan product at 544 nm (Figure 3.3A). The viability of confluent HDFs incubated with a physiological concentration (100 μM) of AA or AA2P for 24 hours was determined with the MTT assay. As depicted in Figures 3.3B and 3.3C, none of the vitamin C supplements reduced the relative viable cell number in GM5399 or in GM5659 HDFs. Viability of control and vitamin C-treated HDFs was also routinely assessed by the trypan blue exclusion assay. Likewise, cell viability was > 95 % in all groups. So, it can be concluded that AA did not have an effect on the viability of nearly confluent HDFs. For cell proliferation studies, HDFs were seeded at a low density (0.33×10^4 cells/cm^2) and incubated with 20, 100 or 500 μM solutions of either AA or AAP or growth medium alone. Medium was changed daily. Control cells were harvested and counted following 0, 3, 5 and 7 days of incubation. HDFs divided exponentially for 5 days and then began to plateau at around the seventh day in culture (Figure 3.4A). Cells incubated with the two vitamin C derivatives were harvested and counted at day 5. Notably, AA, but not AA2P, caused a dose-dependent reduction in the proliferation of HDFs seeded at low cell density (Figure 3.4B). The highest dose of AA caused cell death within a few hours, as indicated by cell shrinkage and blebbing, followed by detachment. The same effect was previously described in chick embryo fibroblasts incubated with sodium ascorbate at a low density (Peterkofsky and Prather 1977).

3.3.3. Effects of vitamin C on DNA damage

Nearly confluent HDFs were exposed to increasing concentrations of H_2O_2 or AA on ice to prevent any DNA repair and processed for the comet assay as described in Materials and Methods (Section 2.8). All treatments were carried out in medium with a minimal amount of serum (0.5 %) in order to avoid interference of the serum constituents. H_2O_2 produced the anticipated dose-response relationship in the comet assay (Figure 3.5A). Vitamin C solutions were prepared immediately before use and incubated at 37 °C for 1 hour in the dark to allow for "auto-oxidation". Only then were the solutions added to cells and incubated on ice for 30 minutes, before processing them for the comet assay. AA caused DNA damage in a dose-dependent manner (Figure 3.5B). An increase in DNA damage was detected with AA concentrations higher than or equal to 100 μM. DNA damage levels induced by 500 μM AA were equivalent to those

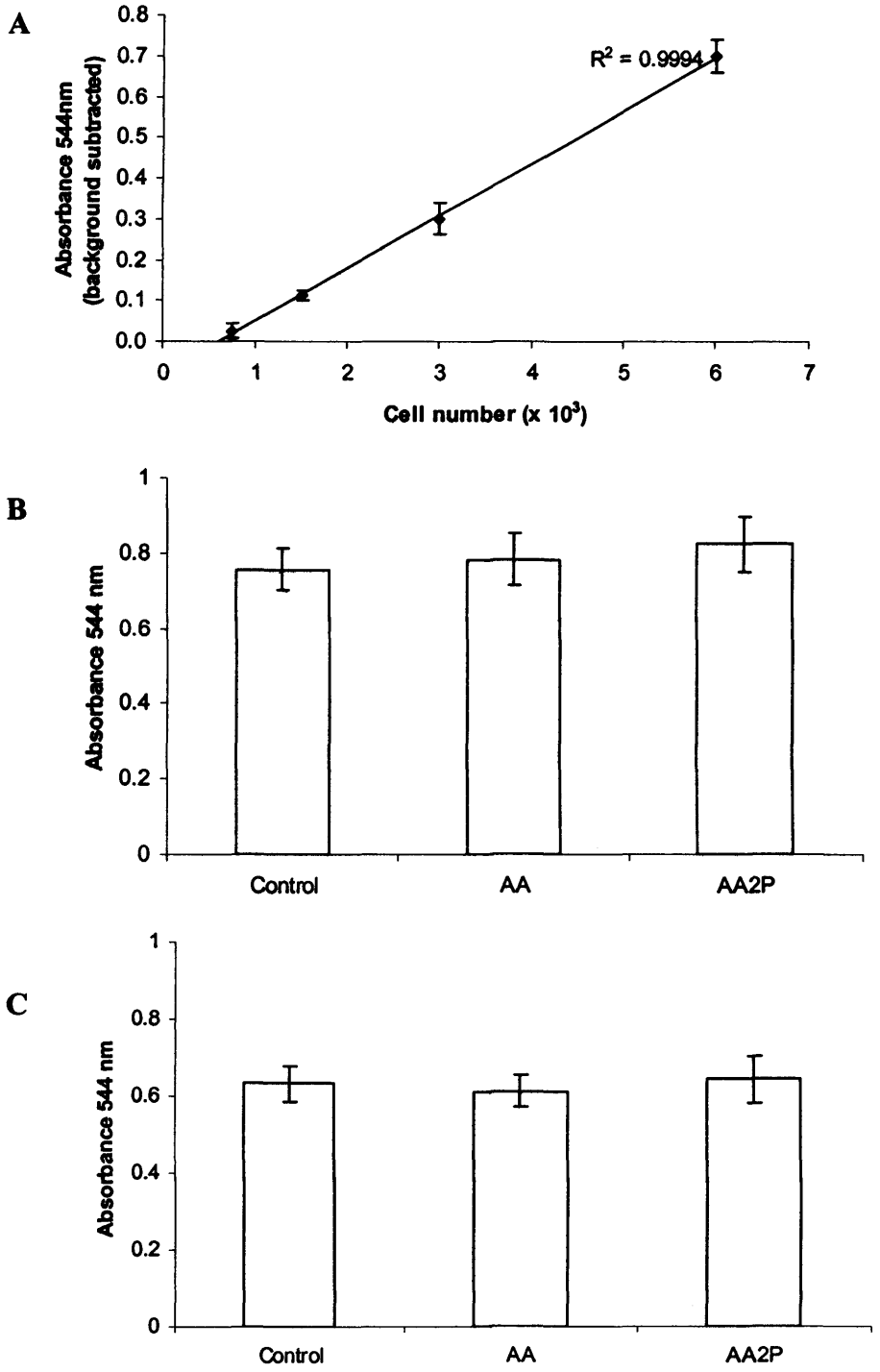


Figure 3.3. Cell viability assessment using the colorimetric MTT assay. A, A 2-fold serial dilution was prepared and HDFs were seeded at different densities (between 6×10^3 and 0.375×10^3 cells per well) in 96-well plates and allowed to attach overnight, before performing the MTT assay. Each value represents the mean ± SD from 8 replicate wells. B, Confluent GM5399 HDFs were incubated with 100 μM AA or AA2P for 24 hours and viability determined with the MTT assay. Each value represents the mean ± SD from 18 replicate wells. C, Confluent GM5659 HDFs were incubated with 100 μM AA or AA2P for 24 hours and viability determined with the MTT assay. Each value represents the mean ± SD from 54 replicate wells.

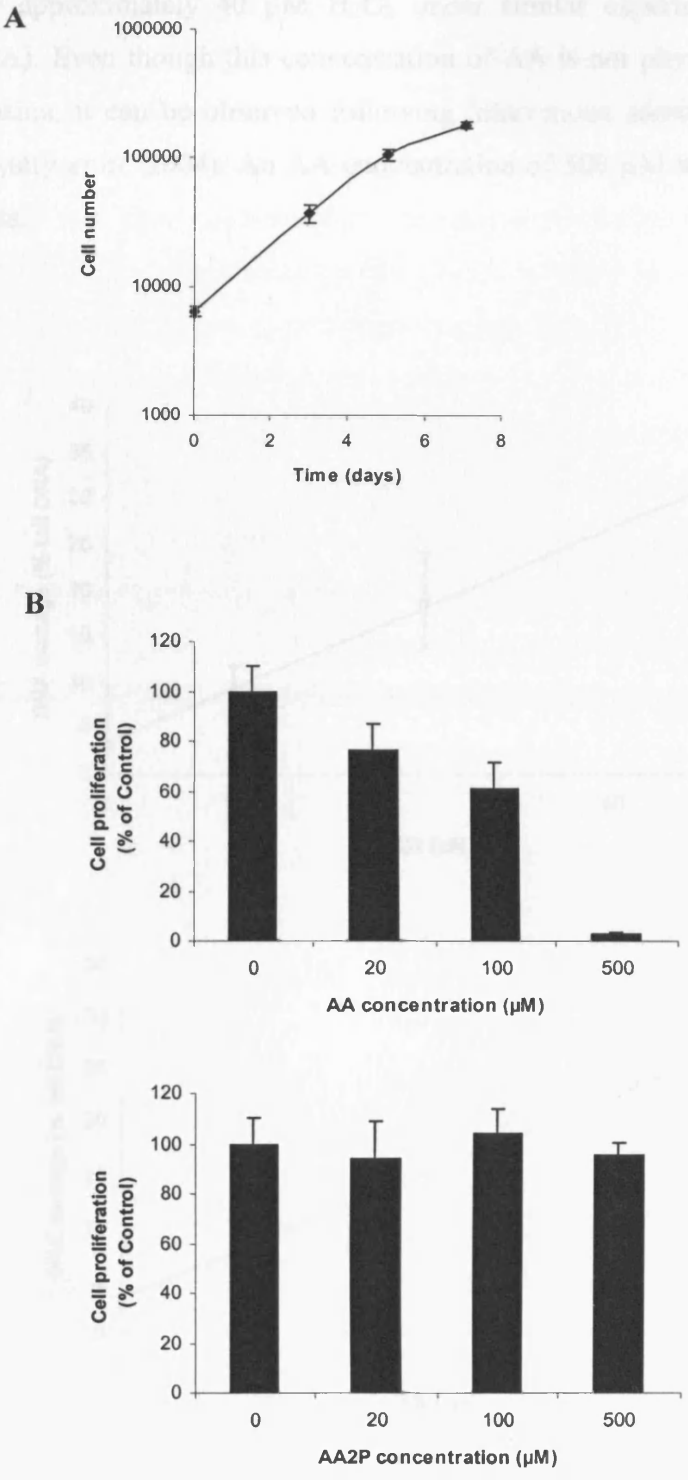


Figure 3.4. Effects of vitamin C on the proliferation of HDFs. A, HDFs seeded at low-density were incubated in growth medium for up to 7 days and counted at 0, 3, 5 and 7 days. Each value represents the mean ± SD from 3 independent samples. B, HDFs seeded at low-density were incubated in the presence of 20, 100 or 500 μM AA or AA2P for 5 days. Medium was changed daily and viable cell number compared with that of control cells. Each value represents the mean ± SD from 3 independent samples.

caused by approximately 40 μM H_2O_2 under similar experimental conditions (see Figure 3.5A). Even though this concentration of AA is not physiologically relevant in human plasma, it can be observed following intravenous administration of high-dose AA (Padayatty *et al.* 2004). An AA concentration of 500 μM was used in subsequent experiments.

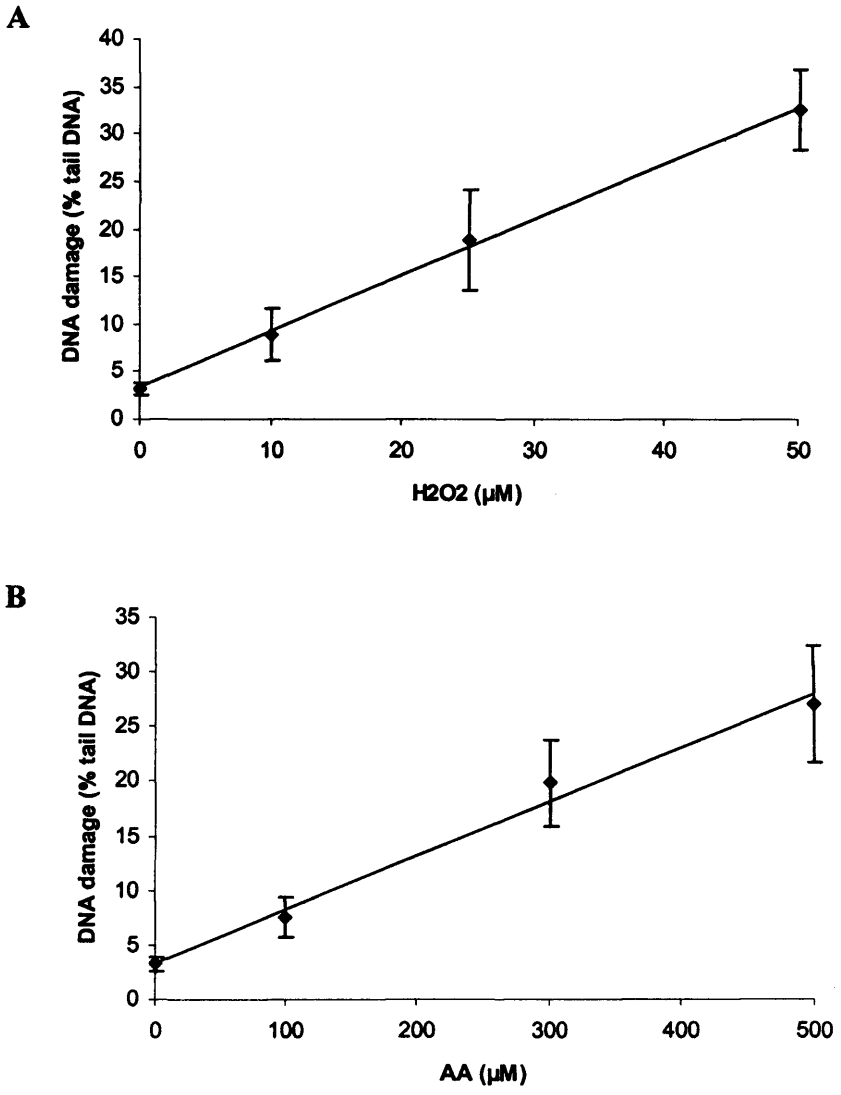


Figure 3.5. H_2O_2- and AA-mediated DNA damage formation in HDFs. A, Cells were incubated with different concentrations of H_2O_2 in low-serum medium for 30 minutes on ice and then processed for the comet assay. Each value represents the mean ± SD from 3 assays. B, Vitamin C solutions were prepared in low-serum medium and incubated at 37 °C in the dark for 1 hour. Cells were then incubated with these solutions for 30 minutes on ice and subsequently processed for the comet assay. Each value represents the mean ± SD from 3 assays.

In contrast to what was described above, the oxidised form of vitamin C, DHA, and the stable AA derivative, AA2P, were not able to cause DNA damage at concentrations up to 1 mM (Figure 3.6). Although some AA may be released from AA2P as a consequence of the activity of serum phosphatases (Chepda *et al.* 2001b), no DNA damage was observed here when AA2P was pre-incubated in complete growth medium (10 % FBS), which indicates that the contribution of serum phosphatases was insignificant. Overall, these experiments suggest that DNA damaging species are produced in the medium during the pre-incubation at 37 °C when the free, reduced form of AA is present. To test this hypothesis, AA solutions were pre-incubated at 37 °C for different periods of time before adding to cells (Figure 3.7). Notably, no DNA damage was detected when AA solutions were prepared and immediately added to cells on ice. However, DNA damage levels were significantly elevated when AA solutions were pre-incubated for 1 or 2 hours at 37 °C. These results suggest that it is a product formed during AA auto-oxidation, rather than AA itself, that is responsible for DNA damage. Longer pre-incubation times (4-8 hours) decreased AA-induced DNA damage substantially, which indicates that the species responsible for DNA damage are only transiently produced and are not stable in solution for long.

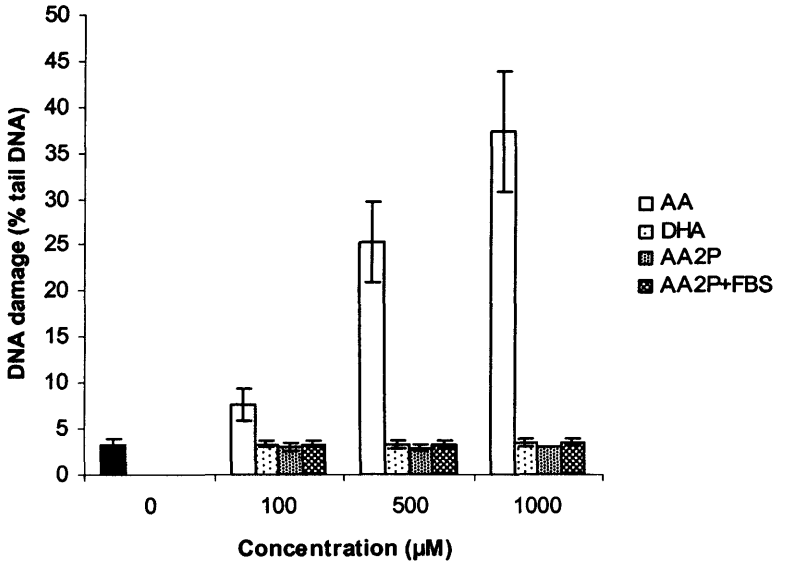


Figure 3.6. Comparison of the ability of AA, DHA and AA2P to produce DNA damage. Vitamin C solutions were prepared in low-serum medium or medium containing 10 % FBS, as indicated, and incubated at 37 °C in the dark for 1 hour. Cells were then incubated with these solutions for 30 minutes on ice and subsequently processed for the comet assay. Each value represents the mean ± SD from 3-7 assays.

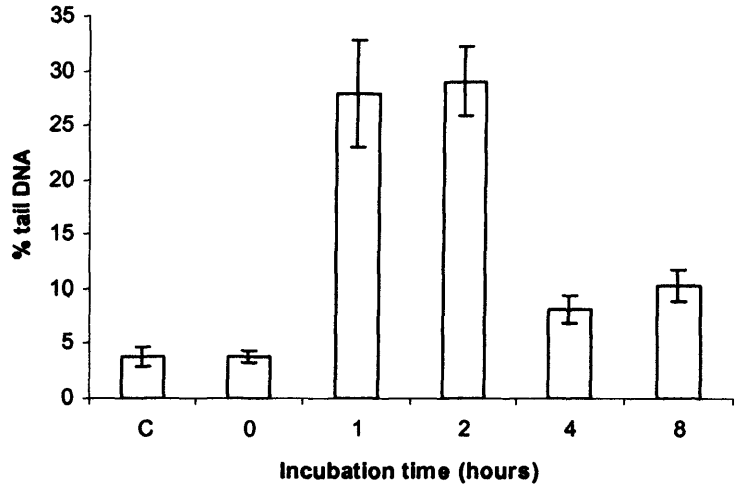


Figure 3.7. Effect of the duration of AA pre-incubation on DNA damage. AA solutions (500 μM) were incubated at 37 °C in the dark for different periods of time. Cells were then incubated with these solutions for 30 minutes on ice and subsequently processed for the comet assay. Each value represents the mean ± SD from 3 independent samples.

Previous reports have shown that AA auto-oxidation in culture medium in aerobic conditions leads to the formation of H_2O_2 (Clément *et al.* 2001; Wee *et al.* 2003). To test the hypothesis that AA-induced DNA damage is due to the formation of H_2O_2 in the culture medium, experiments were performed in which AA solutions were pre-incubated at 37 °C in the presence or absence of catalase (CAT; EC Number: 1.11.1.6), before being added to cells on ice. As shown in Figure 3.8, CAT could completely abolish the AA-induced DNA damage. As the solutions were added to cells on ice, the enzyme would have only been active during the pre-incubation time. Therefore, it can be concluded that the formation of H_2O_2 during the pre-incubation time was essential for the ability of AA to damage the DNA. Furthermore, experiments where AA solutions were incubated at 37 °C and then added to cells on ice in the presence of 25 μM of H_2O_2 showed that the effects of AA and H_2O_2 were additive. This finding is consistent with previous reports indicating that the combination of AA and H_2O_2 additively increases the cytotoxic activity of AA (Sakagami and Satoh 1997a). The effect of SOD during AA incubation was also tested. SOD (200 U/ml; EC Number: 1.15.1.1) on its own did not have an effect on DNA damage and it could only slightly reduce the DNA damage elicited by AA (Figure 3.8).

The experiments described so far suggested that a DNA damaging species, namely H_2O_2, is produced in the medium during the pre-incubation at 37 °C due to the

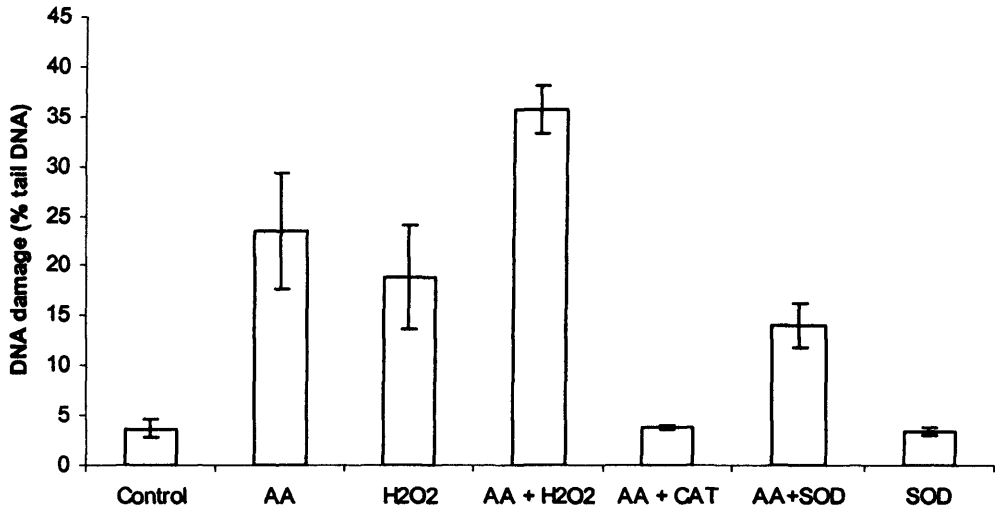


Figure 3.8. Effects of H_2O_2 and antioxidant enzymes on AA-induced DNA damage. AA solutions (500 μM) were incubated at 37 °C in the dark for 1 hour in the presence or absence of either 25 μM H_2O_2, 200 U/ml CAT or 200 U/ml SOD. Cells were then incubated with these solutions for 30 minutes on ice and subsequently processed for the comet assay. Each value represents the mean ± SD from 3-6 independent samples.

auto-oxidation of AA. The auto-oxidation of AA in solution is, in turn, thought to be dependent on the presence of trace levels of metals (Buettner 1988). The contribution of extracellular transition metal ions was therefore investigated by pre-incubating AA at 37 °C in the presence of equimolar amounts of different metal chelators. Whilst the addition of exogenous trivalent (DTPA) and divalent (FZN) metal chelators did not seem to affect the AA-induced DNA damage levels, two iron-specific chelating agents, DFO and *apo*-transferrin, augmented the damage (Figure 3.9). On the other hand, co-incubation with BIP prevented DNA strand breakage completely. However, this was probably due to the ability of this metal chelator to permeate cells quickly and hence chelate intracellular reactive iron during the incubation with AA on ice. Indeed, AA-induced DNA damage was also completely abrogated when cells were pre-incubated with DFO for 14 hours (Figure 3.9), which allows for efficient intracellular accumulation of the chelator (*e.g.* Halliwell 1989 and the results presented in Section 4.2.2 of the present study). Taken together, these results suggest that, whilst the DNA damage elicited by AA solutions is suppressed by effective chelation of intracellular iron, the formation of the damaging species (*i.e.* H_2O_2) in the medium during AA auto-oxidation is not prevented by extracellular transition metal chelating agents.

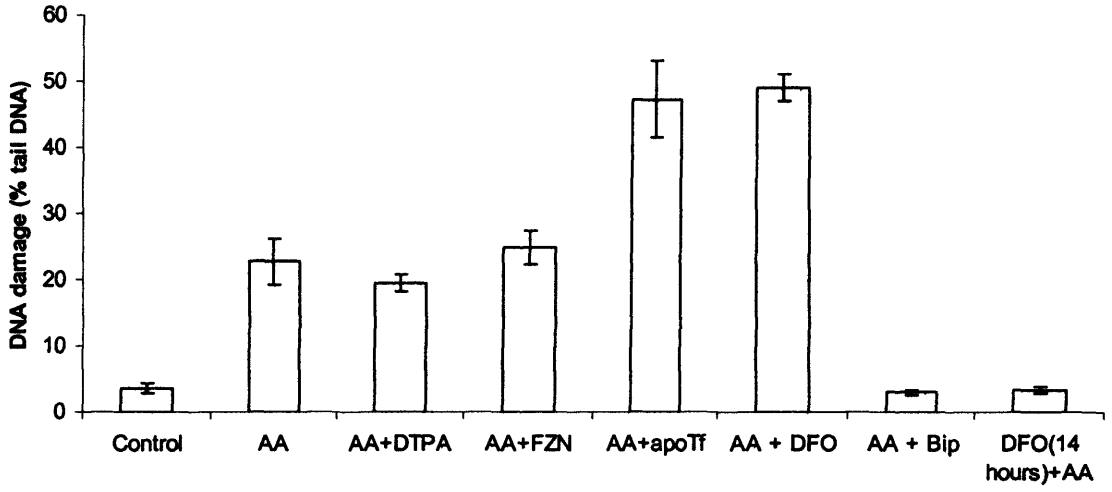


Figure 3.9. Effects of metal chelators on AA-induced DNA damage. AA solutions (500 μM) were incubated at 37 °C in the dark for 1 hour in the presence or absence of 50 μg/ml *apo*-transferrin or 500 μM DFO, BIP, DTPA or FZN. Cells were then incubated with these solutions for 30 minutes on ice and subsequently processed for the comet assay. The last treatment refers to cells that were pre-loaded with 300 μM DFO for 14 hours and thoroughly washed before exposure to AA. Each value represents the mean ± SD from 3-10 independent samples.

3.4. Discussion

3.4.1. The involvement of H_2O_2 in the pro-oxidant effect of vitamin C

The present study confirmed that AA is unstable in culture media. Although cellular uptake of AA may contribute to the decrease of extracellular AA with time, other studies have demonstrated that most of this effect is attributed to the auto-oxidation of AA in the culture medium (Peterkofsky 1972; Franceschi *et al.* 1995; Chepda *et al.* 2001a).

In this work, physiological concentrations of AA were not toxic to confluent HDFs, but they inhibited the proliferation of cells treated at low density. This is in agreement with previous data in the literature. In fact, studies reporting that AA stimulates the proliferation of cultured HDFs were generally performed with confluent cells (Hata *et al.* 1988; Chan *et al.* 1990; Chepda *et al.* 2001a), whereas studies showing a cytotoxic effect have used cells at low density and/or high (millimolar) concentrations of AA (Peterkofsky and Prather 1977; Michiels *et al.* 1990; Peterszegi *et al.* 2002). On the other hand, the stable vitamin C derivative, AA2P, was not cytotoxic in any circumstance, not even when supplied at a high dose (500 μM). The results agree with previous evidence that the cytotoxic activity of AA is exclusive to those AA derivatives

that auto-oxidise in culture medium (*e.g.* L-ascorbic acid and sodium-L-ascorbate), whereas stable AA derivatives (*e.g.* L-ascorbic acid-2 phosphate magnesium salt and L-ascorbic acid 2-sulfate) are not toxic (reviewed by Sakagami and Satoh 1997b). It is widely accepted that AA is unstable in aqueous media and in the presence of oxygen it auto-oxidises to DHA, generating AFR and H_2O_2. The latter is thought to be responsible for the cytotoxic activity of AA (Peterkofsky and Prather 1977; Galloway and Painter 1979; Prasad *et al.* 1979; Peterszegi *et al.* 2002).

To date, studies employing the alkaline comet assay have shown that in some, but not all, conditions, AA causes DNA strand breakage in cultured cells. For instance, whilst *ex vivo* exposure of human lymphocytes to AA concentrations of up to 200 μM failed to induce any DNA damage in one study (Szeto and Benzie 2002), a dose-dependent increase in DNA damage was observed in another study between 200-5000 μM (Anderson *et al.* 1994). Furthermore, sodium ascorbate (25-100 μM) caused DNA strand breakage in HDFs and lymphocytes (Singh 1997). The present study confirmed that AA causes DNA damage with a clear dose-related response. DNA damage formation reported in the present study required higher concentrations of AA than those employed by Singh (1997), which may be due to differences in the experimental protocols. In the latter study, cells were incubated with sodium ascorbate, which auto-oxidises more rapidly than L-ascorbic acid in culture medium (Sakagami and Satoh 1997b). In addition, the author treated HDFs at 4×10^3 cells per cm^2, whereas nearly confluent monolayers ($> 2\times10^4$ cells per cm^2) were treated in the present study. It is known that the levels of DNA damage caused by H_2O_2 (which is proposed to be involved in DNA damage formation, as discussed below) are dramatically reduced in confluent cell cultures (Duthie and Collins 1997). The model used in the present study is, however, closer to the *in vivo* situation.

To clarify the pro-oxidant activity of vitamin C in cell culture models, vitamin C solutions were incubated in aerobic conditions at 37 °C for different periods of time before being added to cells. HDFs were then incubated with these solutions at 0 °C, which suppresses AA accumulation (Butler *et al.* 1991). Consequently, any genotoxic effects could be attributed to a product of AA auto-oxidation capable of entering cells by passive diffusion, rather than to AA itself. With this different approach, it was possible to show here that only when vitamin C solutions were incubated at 37 °C to allow for auto-oxidation before adding to cells, AA, but not the oxidised form of the vitamin, DHA, or the stable derivative, AA2P, was able to damage the DNA in a dose-

dependent manner. The pro-oxidant effect was maximal within the first 2 hours of AA incubation and diminished at longer incubation times. Taken together, these results suggest that DNA damage is not caused by vitamin C itself but rather by products of AA oxidation formed during the first two hours of incubation. Experiments where CAT was present during the pre-incubation period identified extracellular H_2O_2 as the mediator of DNA damage formation. As described before (see Section 1.3.3), H_2O_2 is poorly reactive and it can easily reach the nuclear compartment where, in the presence of intracellular reactive iron, it can yield highly reactive oxygen species that damage the DNA (Halliwell and Gutteridge 1999). The dependence on intracellular reactive iron was confirmed in the present study by the fact that a long incubation with DFO abolished the AA-mediated DNA damage.

3.4.2. Implications for the use of vitamin C in cell culture studies

The formation of extracellular H_2O_2 may explain many of the effects of vitamin C on cultured cells (Halliwell *et al.* 2000), including its cytotoxicity (Peterkofsky and Prather 1977; Galloway and Painter 1979; Prasad *et al.* 1979; Peterszegi *et al.* 2002). Regression analysis of the dose-response curves of DNA damage formation induced by AA or H_2O_2 indicates that, under the experimental conditions employed herein, 1 mM AA induced an amount of DNA damage equivalent to that induced by 56 μM H_2O_2. This is in agreement with previous studies. Wee *et al.* (2003) measured H_2O_2 in cell medium and observed that approximately 60 μM of H_2O_2 were generated from 1 mM L-ascorbic acid within 1 hour of incubation in Dulbecco's MEM at 37 °C. Likewise, 1 mM L-AA induced apoptosis in acute myeloid leukaemia cells to an extent similar to that caused by incubation with 50 μM H_2O_2 (Park *et al.* 2004). The formation of extracellular H_2O_2 may explain the loss of cell viability observed at low cell density, especially following exposure to high (millimolar) doses of AA. However, 100 μM AA induced an amount of DNA damage lower than that induced by 10 μM H_2O_2, which is likely to be tolerable. Therefore, the pro-oxidant effect is expected to be minimal when AA is supplied to confluent cells at physiologic concentrations (*i.e.* equal to or lower than 100 μM). The increased resistance to H_2O_2 at higher cell density may be explained by leakage of CAT from cells into the medium (Halliwell 2003). AA2P, on the other hand, did not have a pro-oxidant effect and did not affect cell viability, even when HDFs were treated at a low cell density. Therefore, studies of the intracellular effects of

vitamin C, which require the use of cells at low density, would benefit from the use of a stable vitamin C derivative like AA2P.

In the recent years, AA has been employed in genotoxicity studies as a supposed antioxidant in order to clarify the mechanisms of action of numerous test agents. These include 5-nitroimidazoles (Re *et al.* 1997), cadmium chloride (Blasiak *et al.* 2000), nickel chloride (Wozniak and Blasiak 2002), lead acetate (Wozniak and Blasiak 2003), a cisplatin conjugate (Blasiak and Kowalik 2001), amsacrine (Blasiak *et al.* 2003a), alloxan (Blasiak *et al.* 2003b), vanadyl sulphate (Wozniak and Blasiak 2004) and *N*-nitroso compounds (Robichova and Slamenova 2002). For example, Blasiak *et al.* (2000) incubated human lymphocytes with cadmium chloride in the presence or absence of sodium ascorbate (20-100 μM) for 1 hour at 37 °C and reported that vitamin C increased the genotoxic effects of cadmium. However, analysis of their results shows that vitamin C *per se* increases DNA damage. Hence the effects of vitamin C and cadmium chloride were most likely additive, rather than synergistic. In another study, sodium ascorbate (50 μM) was reported to decrease the DNA strand breakage induced by alloxan (Blasiak *et al.* 2003b), but an increase in baseline DNA damage was also apparent. Thus, the results presented herein have strong implications for genotoxicity studies, especially those using ascorbic acid as an antioxidant to counteract the effects of other test compounds.

3.4.3. The involvement of metal ions in the pro-oxidant effect of vitamin C

The oxidation of AA in the presence of oxygen can either occur spontaneously or be catalysed by transition metal ions. The former involves the one-electron oxidation of ascorbate anion by molecular oxygen, whereas the latter involves the reaction of oxygen with a metal-ascorbate complex (Khan and Martell 1967). In fact, chelating agents were shown to slow the oxidation of AA catalysed by iron (DTPA or DFO) or copper (DTPA) in phosphate buffers at neutral pH (Buettner 1986). It has been argued that the auto-oxidation of AA in solution is a consequence of trace levels of metal ion (iron and to a lower extent copper) contamination in most solutions employed for research (Buettner 1988) and would therefore be of limited physiological significance, as metals are efficiently sequestered in biological fluids from healthy individuals (Miller *et al.* 1990). However, results presented here showed that the transient pro-oxidant effect of AA could not be blocked by the addition of the metal chelators *apo*-transferrin, DFO, FZN or DTPA. It is worth noting that the endocytosis of DFO and *apo*-transferrin

is inhibited at 0 °C and likewise FZN and DTPA are not expected to enter cells during the short incubation on ice due to their hydrophilic nature (Porter 1989; Parkes *et al.* 1997). Therefore, these agents would only account for the chelation of extracellular metals in the present experimental conditions. These data suggest that the genotoxicity of AA solutions is due to AA's spontaneous, rather than metal-catalysed, oxidation in the presence of oxygen.

Intriguingly, the addition of *apo*-transferrin and DFO led to an increase in AA-mediated DNA damage. A possible explanation is that, in the absence of the chelators, some of the H_2O_2 formed from AA auto-oxidation would be lost before entering cells by reacting with any contaminating or serum-derived iron. An excess of either *apo*-transferrin or DFO would inhibit the availability of ferric iron and thus spare H_2O_2 loss in the medium. As a consequence, more H_2O_2 would enter cells and damage the DNA in the presence of intracellular reactive iron. It is possible that, unlike *apo*-transferrin and DFO, the addition of DTPA did not increase DNA damage because, in the presence of AA, most iron would be present as Fe^{2+}-DTPA and these chelates are known to catalyse hydroxyl radical formation from H_2O_2 (Cohen and Sinet 1982). The effect of DFO and *apo*-transferrin is similar to that reported by Byrnes (1996), who observed that chelation of extracellular iron with non-permeant chelators (bathophenantholine disulfonic acid or 1,10-phenanthroline) increased H_2O_2-induced DNA strand breakage in HL-60 cells. This is further supported by the work of Sakagami *et al.* (1997), who reported that a 1-hour exposure to high (millimolar) concentrations of AA was toxic to human promyelocytic leukemic HL-60 cells. The addition of micromolar to millimolar concentrations of DFO during the 1-hour exposure did not inhibit but rather enhanced the cytotoxic activity of AA in a dose-dependent way. Taken together, the two studies mentioned above and the present data show that high concentrations of AA can be toxic even in iron-deficient medium and that iron chelation may enhance the cytotoxicity and genotoxicity of AA.

3.4.4. Possible relevance of vitamin C auto-oxidation *in vivo*

As the pro-oxidant effect described above can occur in the absence of catalytically active metal ions, it can be speculated that vitamin C may promote H_2O_2 formation in biological fluids. In this respect, it is likely that the cytotoxicity and genotoxicity associated with the production of H_2O_2 would only be relevant in the case of exposure to high (millimolar) concentrations of ascorbate, as low concentrations of

H_2O_2 would be reasonably well tolerated by cells. However, plasma AA levels are generally well regulated by urinary excretion (Levine *et al.* 1996) and it is well accepted that oral supplements only modestly elevate plasma AA concentration when compared to the effect of a vitamin C-rich diet. In fact, plasma AA deriving from oral supplements cannot exceed ~100-150 μM, even when the highest tolerable doses are employed, because of limited intestinal absorption (Olson and Hodges 1987). Nevertheless, recent pharmacokinetics studies with human volunteers demonstrated that plasma AA levels can reach concentrations as high as 15 mM immediately after the intravenous administration of high doses (grams) of vitamin C (Padayatty *et al.* 2004). This is followed by a rapid decrease in plasma AA, whereby only 60 % and 20 % of the initial concentration are present at 2 and 6 hours post intravenous administration, respectively. AA disappearance is thought to occur mainly by efficient renal filtration and excretion, but AA oxidation may also have a significant contribution. Indeed, AFR can be detected in the circulating blood of rats (Wang *et al.* 1992) and humans (Galley *et al.* 1996) given a high intravenous dose of AA. Increased levels of AFR are considered a marker of *in vivo* oxidative stress and have been measured in UV-irradiated skin, ischemic and reperfused hearts, during sepsis and xenobiotic metabolism (Buettner and Jurkiewicz 1996). There is in fact some evidence that intravenous AA supplements may elicit a pro-oxidant effect *in vivo*. High doses of sodium ascorbate (about 5 g) are often administered intravenously during EDTA chelation therapy in the treatment of diabetes or cardiovascular disease. A recent study showed that standard EDTA chelation therapy (containing AA) caused a rapid and transient increase in markers of oxidative stress such as plasma disulfide and MDA, and DNA damage assessed with the alkaline comet assay. Notably, when vitamin C was removed from the EDTA chelation cocktail, there were no pro-oxidant effects (Hininger *et al.* 2005). Finally, it has recently been proposed that the use of high (grams) intravenous doses of AA may be a clinically useful way to deliver H_2O_2 to tissues and thereby selectively kill cancer cells (Chen *et al.* 2005), which are thought to have lower levels of endogenous CAT activity (Alcain and Buron 1994). In fact, Chen *et al.* (2005) investigated the effects of 1-hour incubation with high-dose AA on the viability of 4 normal cell lines and 9 cancer cell lines. Whilst normal cells were resistant to AA concentrations up to 20 mM, survival of cancer cells was reduced to different degrees by AA concentrations often below 5 mM, *i.e.* at doses that can be easily achievable from intravenous infusion. DHA did not kill cells, despite causing a similar accumulation of intracellular AA. Moreover, cell death

was inhibited by H_2O_2 scavengers but not by metal chelators, as observed in the DNA damage studies reported herein. The authors measured H_2O_2 formation in the medium during AA incubation. During 1-hour incubation at 37 °C, H_2O_2 concentration increased with time and with AA dose and depended on the presence of 0.5-10 % serum. Finally, a linear relationship was found between H_2O_2 formation and AFR concentration, a product of AA oxidation.

3.5. Summary

AA is unstable in cell culture media. Physiological concentrations of AA ($\leq$100 μM) inhibited the proliferation of HDFs at low-density but were not toxic to confluent cells. At concentrations $\geq$100 μM, AA caused DNA strand breakage in confluent HDFs in a dose-dependent manner. However, this pro-oxidant effect is thought to be minimal when AA is supplied to confluent cells at physiologic concentrations (*i.e.* equal to or lower than 100 μM). AA2P did not have a pro-oxidant effect and did not affect cell viability, even when HDFs were treated at a low cell density. Therefore, studies of the cellular effects of vitamin C that require the use of cells at low density would benefit from the use of a stable vitamin C derivative like AA2P.

With the experimental design adopted herein, it was possible to show that the genotoxic mechanism of AA does not require its intracellular accumulation. Instead, it involves the transient production of H_2O_2 in the cell medium from AA auto-oxidation, the passive diffusion of H_2O_2 through the cell membrane and its reaction with intracellular labile iron, to generate more reactive oxygen species and ultimately damage the DNA. Importantly, the genotoxic effect of AA was independent of the presence of extracellular catalytically active metal ions. Consequently, it can be anticipated that the pro-oxidant effect of AA described above may occur *in vivo* immediately after administration of high-dose AA.

CHAPTER IV

EFFECTS OF VITAMIN C ON OXIDATIVE DNA DAMAGE AND CELL INJURY IN HUMAN FIBROBLASTS

4.1. Specific aims of study

Despite numerous human studies performed to date, an association between plasma or cellular AA and levels of oxidative DNA damage has not been established. Whilst some studies have shown a protective effect, others have suggested that, at least in some conditions, AA supplements may be deleterious. In this respect, it is has been speculated that AA may promote iron-mediated oxidative damage. The aim of this study was to investigate whether intracellular AA enrichment could protect or sensitise human cells against DNA damage and cell death caused by a naturally occurring ROS species (H_2O_2). In particular, the possibility that intracellular AA promotes iron-mediated oxidative stress in normal human cells was evaluated. The effects of AA pre-incubation on H_2O_2-induced DNA damage and cell death were studied using HDFs. The stable vitamin C derivative AA2P was employed to ensure that the effects observed herein could not be attributed to AA auto-oxidation in the medium.

4.2. Results

4.2.1. Intracellular accumulation of AA in HDFs

The concentration of intracellular AA in HDFs was determined by HPLC with electrochemical detection, as described in Materials and Methods (Section 2.12). A representative calibration curve is shown in Figure 4.1A. Without supplementation, AA was not detected in the cells employed in this study. Thus it can be assumed that any AA originally present in these cells has been completely lost due to extended serial culture. When incubated with AA or a stable vitamin C derivative, AA2P, HDFs were able to accumulate AA intracellularly in a time-dependent manner (Figure 4.1B). Cellular uptake of AA was slightly faster than that of AA2P. As previously described, AA2P is stable in culture media and hydrolysed by membrane or intracellular phosphatases to AA, leading to an accumulation of intracellular AA (Furumoto *et al.* 1998; Savini *et al.* 1999). Similar intracellular concentrations of AA were achieved following 12 hours of incubation with either AA or AA2P. Moreover, intracellular AA accumulation from AA2P was dose-dependent (Figure 4.1C).

4.2.2. Effects of vitamin C on H_2O_2-induced DNA damage

DNA damage was measured using the alkaline comet assay. In the assay, cells are lysed and the nucleoid bodies electrophoresed under alkaline conditions (pH > 13). Undamaged DNA remains supercoiled and hardly migrates during the electrophoresis.

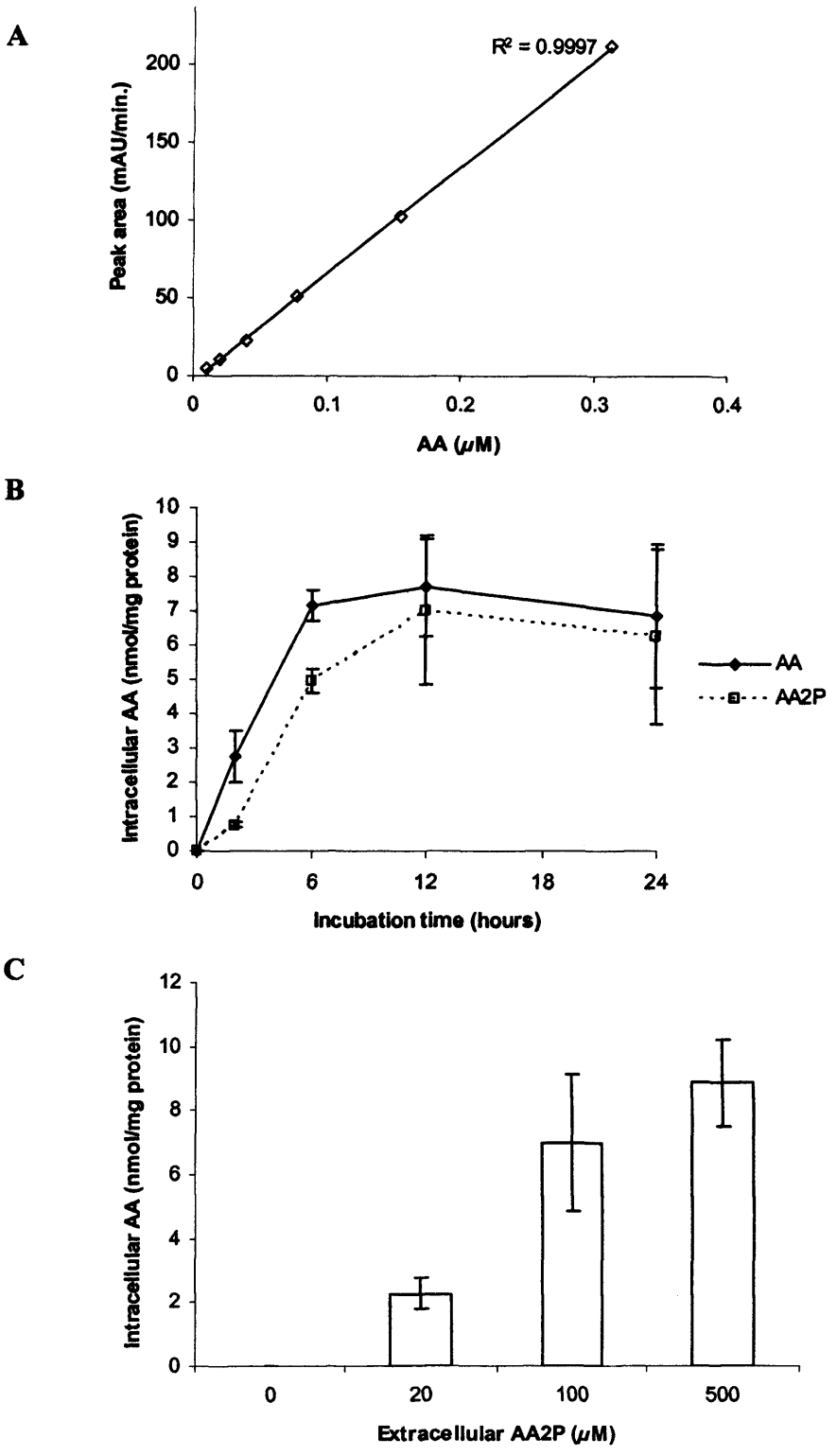


Figure 4.1. Intracellular accumulation of AA in HDFs. Intracellular AA concentration was determined by HPLC-EC and normalised for total protein. A, Example of a calibration curve generated using external AA standards. B, HDFs were incubated with 100 μM AA or AA2P for 0, 2, 6, 12 or 24 hours. Results are the mean ± S.D. from 3 separate determinations. C, HDFs were incubated with 0, 20, 100 or 500 μM AA2P for 12 hours. Results are the mean ± S.D. from 3 separate determinations.

However, the presence of DNA damage in the form of strand breaks or alkali-labile sites relaxes the supercoiled DNA and allows it to migrate during electrophoresis, leading the formation of a 'tail' (Collins 2004). Cells were exposed to a physiologically relevant oxidant insult (H_2O_2) on ice to avoid enzymatic DNA strand breakage as a consequence of DNA repair or the activation of intracellular endonucleases, as well as any possible interference of peroxide degrading enzymes. Moreover, treatments with H_2O_2 were performed in the presence of a minimal amount of serum (0.5 % FBS), which is known to reduce the effect of H_2O_2 on DNA damage (*e.g.* Anderson *et al.* 1994). As expected, H_2O_2 caused a dose-dependent increase in comet tail formation (see Section 3.2.2). To investigate the effect of intracellular AA on oxidative DNA damage, HDFs were pre-incubated with 100 μM AA for approximately 12 hours to allow for efficient intracellular accumulation of AA and then exposed to 50 μM H_2O_2. AA pre-incubation on its own did not cause any detectable DNA damage but it exacerbated H_2O_2-induced DNA damage, as indicated by a clear increase in tail formation (Figure 4.2).

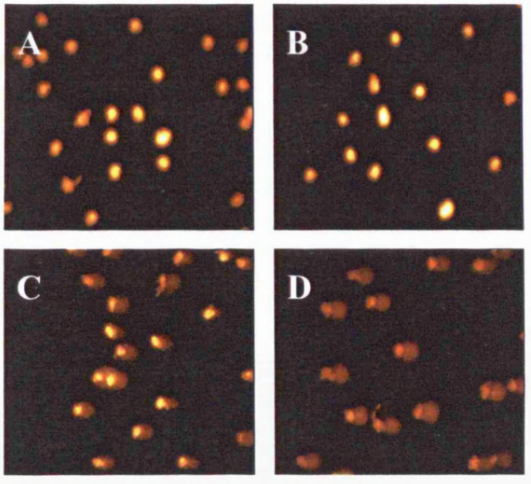


Figure 4.2. Effect of AA pre-incubation on H_2O_2-induced DNA damage in GM5399 HDFs. Cells were incubated in the presence or absence of 100 μM AA for 12 hours before exposure to 50 μM H_2O_2 or medium alone on ice for 30 minutes. Subsequently, cells were subjected to alkaline comet assay analysis as described in Materials and Methods. (A) Appearance of control (untreated) fibroblasts. (B) Appearance of fibroblasts incubated with AA. (C) Appearance of H_2O_2-treated fibroblasts. (D) Appearance of AA-pre-incubated and H_2O_2-treated fibroblasts.

The experiments described above were performed with GM5399 HDFs. To investigate if the effects of AA were specific to this cell line, similar experiments were performed using fibroblasts obtained from a different healthy human donor, GM5659. As for the GM5399 fibroblasts, incubation with 100 μM AA for 12 hours failed to increase the baseline levels of DNA damage when comparing with control cells, but H_2O_2-induced DNA damage was significantly higher in cells that were pre-incubated with AA (Figure 4.3). To investigate if the exacerbating effect of AA on H_2O_2-induced DNA damage was exclusive to fibroblast cell lines, similar experiments were performed with cells of distinct lineage, namely human breast epithelial cell lines HBL-100 and MCF-7. In these experiments, exponentially growing cells were incubated in the presence or absence of vitamin C (supplied as AA or AA2P) for 6 hours before exposure to H_2O_2. Preliminary experiments showed that the levels of H_2O_2-induced DNA damage were nearly 7-fold higher in MCF-7 cells than in HBL-100 (Figure 4.4). Whilst baseline DNA damage levels of cells pre-incubated with either AA or AA2P were similar to those of control cells, higher levels of H_2O_2-induced damage were found following pre-incubation with AA in both epithelial breast cell lines. These results show that the effects of AA presented above are not exclusive to HDFs. Subsequent experiments were performed with GM5399 HDFs, unless otherwise stated.

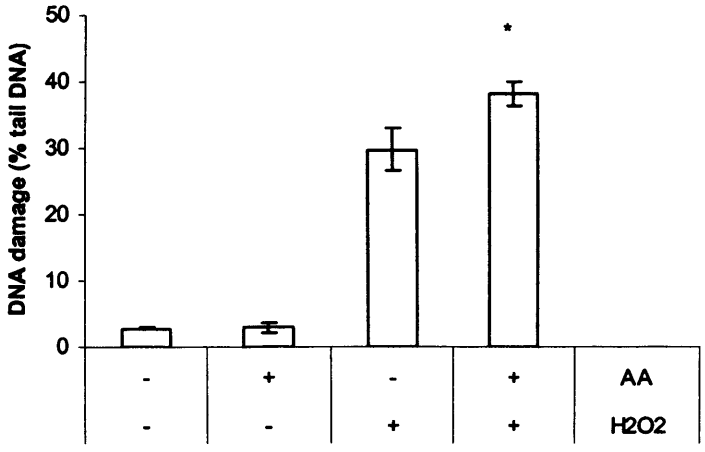


Figure 4.3. Effect of AA pre-incubation on H_2O_2-induced DNA damage in GM5659 HDFs. Cells were incubated in the presence or absence of 100 μM AA for 12 hours before exposure to 50 μM H_2O_2 or medium alone on ice for 30 minutes. Subsequently, cells were subjected to alkaline comet assay analysis as described in Materials and Methods. Results are the mean ± S.D. from 3 separate determinations. * $P < 0.05$ *versus* H_2O_2 treatment without AA pre-incubation.

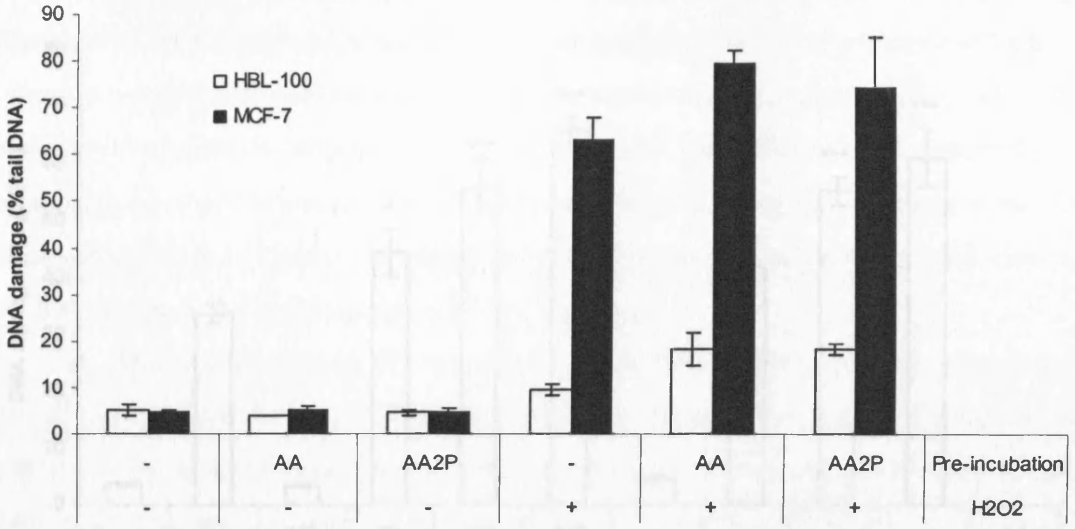


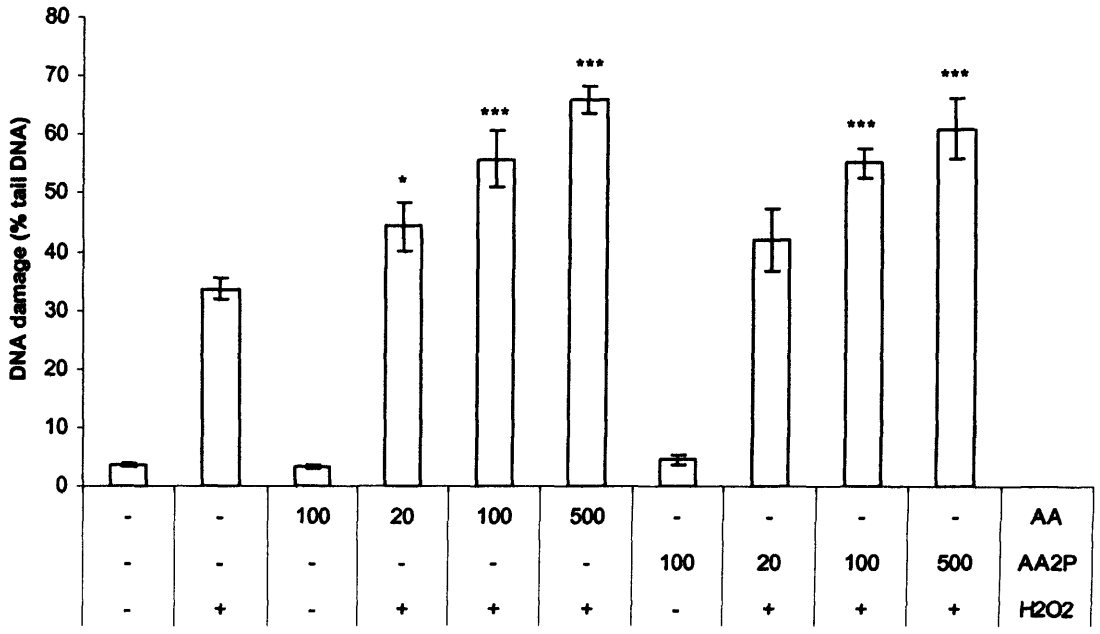
Figure 4.4. Effect of vitamin C pre-incubation on H_2O_2-induced DNA damage in human breast epithelial cells. MCF-7 and HBL-100 cells were incubated in the presence or absence of 100 μM AA or AA2P for 12 hours before exposure to 50 μM H_2O_2 or medium alone on ice for 30 minutes. Subsequently, cells were subjected to alkaline comet assay analysis as described in Materials and Methods. Results are the mean ± standard error from 2 separate determinations.

To investigate the dose-dependence of the effect of vitamin C on H_2O_2-induced DNA damage, HDFs were pre-incubated with different concentrations of AA or the stable vitamin C derivative, AA2P, for 12 hours before exposure to 50 μM H_2O_2. Pre-incubation with AA and AA2P (20-500 μM) significantly enhanced the DNA damage caused by H_2O_2 in a dose-dependent manner (Figure 4.5A). It is noteworthy that the exacerbating effect of vitamin C was significant even when low, physiologically relevant concentrations of AA were employed (20-100 μM).

The effect of AA2P depicted above indicates that the vitamin C-mediated exacerbation of oxidative DNA damage is not related with the extracellular auto-oxidation of AA (discussed in Chapter 3). Furthermore, a clear linear relationship was observed between the measured intracellular levels of AA attained when cells were incubated with increasing doses of AA2P (0, 20, 100 or 500 μM) and the corresponding levels of H_2O_2-induced DNA damage ($r^2 = 0.997$, P=0.001) (Figure 4.5B). This suggests that the effect of vitamin C on H_2O_2-induced DNA damage is due to the intracellular accumulation of AA.

To assess the effect of AA supplementation on cellular DNA repair capacity, AA-loaded and control cells were exposed to H_2O_2 on ice as before, washed with PBS

A



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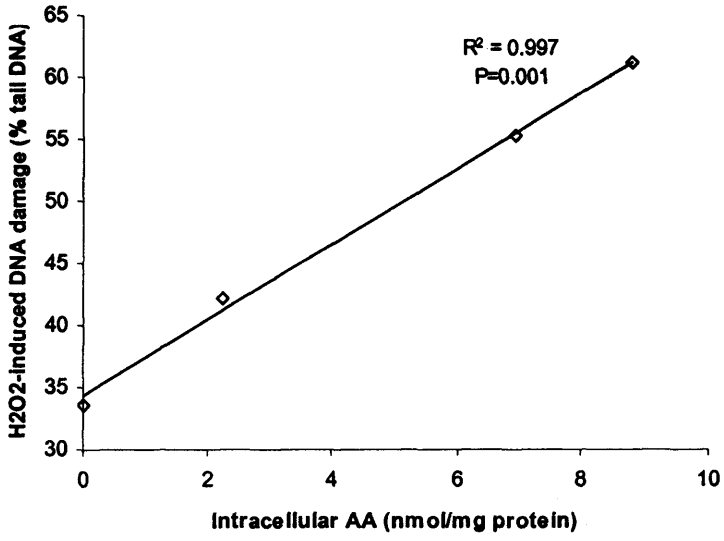


Figure 4.5. Dose-dependence of the effect of vitamin C on H_2O_2-induced DNA damage. A, HDFs were pre-incubated with different concentrations (20-500 μM) of either AA or AA2P for 12 hours before exposure to 50 μM H_2O_2. DNA damage was determined with the alkaline comet assay. Results are the mean ± S.D. from 3 separate determinations. * $P < 0.05$; *** $P < 0.001$ *versus* H_2O_2 treatment without AA or AA2P pre-incubation. B, The intracellular AA concentrations achieved when HDFs were incubated with 0, 20, 100 or 500 μM AA2P reported in Figure 4.1C were plotted against the respective values of H_2O_2-induced DNA damage shown in Figure 4.5A. The relationship between the two variables was determined by linear regression analysis.

and subsequently incubated in complete culture medium at 37 °C for 0, 15, 30 or 60 minutes (Figure 4.7). Nearly 80 % of the damage was repaired within the first 30 minutes post-exposure to H_2O_2 and most of the remaining damage was repaired by the end of the 60 minutes incubation period. H_2O_2-induced DNA damage was repaired with the same kinetics in control and AA-loaded cells, indicating that DNA lesions measured by the comet assay in control and AA-loaded cells are of the same nature and that AA does not change the cellular repair capacity.

To investigate whether H_2O_2-induced DNA damage required the presence of intracellular catalytic iron, the 12 hours pre-incubation with AA was performed in the presence of the specific iron chelator DFO at a final concentration of 300 μM (Figure 4.8A). H_2O_2 did not damage the DNA of cells that had been pre-incubated with DFO. This shows that H_2O_2 is only able to damage DNA in the presence of non-chelated iron. Likewise, intracellular iron chelation with DFO abolished the exacerbating effect of AA on H_2O_2-induced DNA damage. To ensure that the stimulatory effect of AA on H_2O_2-induced DNA damage was also dependent on intracellular iron, rather than iron present in the medium, the AA pre-incubation was performed in the presence of an excess of *apo*-transferrin (50 μg/ml), which chelates extracellular free iron. As depicted in Figure 4.8B, the exacerbating effect of AA on H_2O_2-induced DNA damage was not affected by the addition of exogenous *apo*-transferrin.

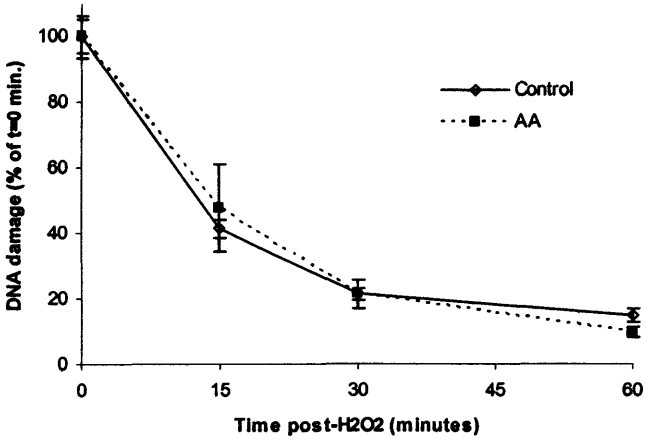
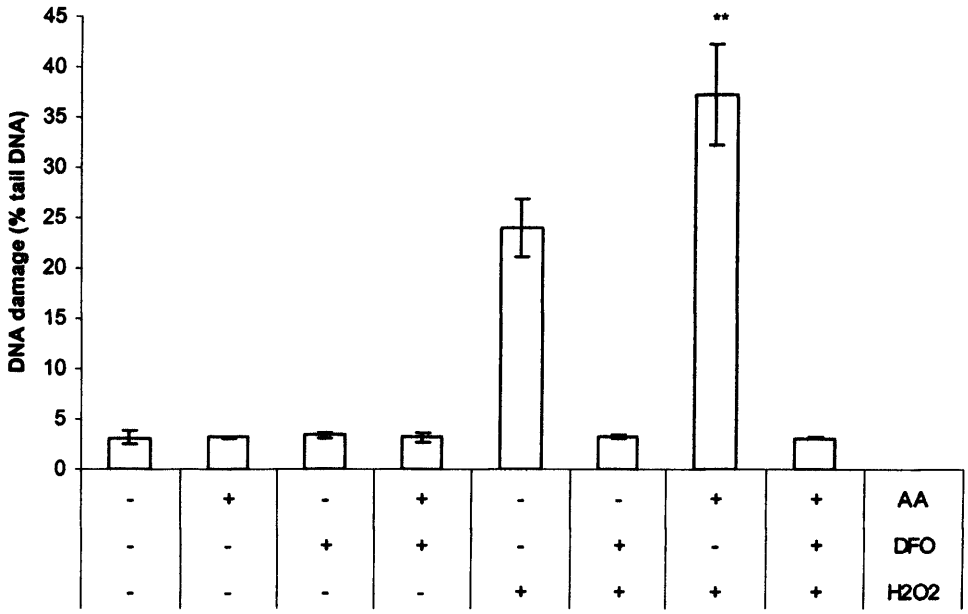


Figure 4.7. Repair kinetics of H_2O_2-induced DNA damage in HDFs. Cells were incubated with 100 μM AA or medium alone for 12 hours before exposure to 50 μM H_2O_2 and subsequently incubated in complete culture medium at 37 °C for 0, 15, 30 or 60 minutes. DNA damage was determined with the alkaline comet assay and expressed as a percentage of the initial levels of damage. Results are the mean ± S.D. from 3 separate determinations.

A



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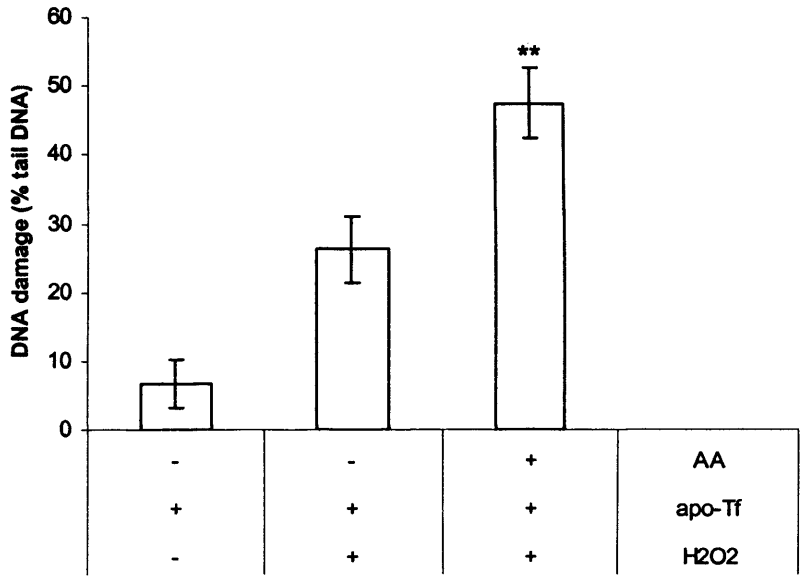


Figure 4.8. Effect of iron chelation on H_2O_2-induced DNA damage in HDFs. A, Cells were incubated with 100 μM AA or medium for 12 hours in the presence or absence of DFO before exposure to 50 μM H_2O_2. DNA damage was determined with the alkaline comet assay. Results are the mean ± S.D. from 3 separate determinations. ** $P < 0.01$ *versus* H_2O_2 treatment without AA pre-incubation. B, Cells were incubated with 100 μM AA or medium alone for 12 hours in the presence of 50 μg/ml *apo*-transferrin before exposure to 50 μM H_2O_2. DNA damage was determined with the alkaline comet assay. Results are the mean ± S.D. from 3 separate determinations. ** $P < 0.005$ *versus* H_2O_2 treatment without AA pre-incubation.

As the DNA damaging effect of H_2O_2 was dependent on intracellular iron, it can be speculated that AA could augment this effect by promoting intracellular Fenton chemistry. To assess this hypothesis, experiments were conducted to ascertain whether iron supplementation could further augment the exacerbating effect of AA on H_2O_2-induced DNA damage. Nearly confluent HDFs were incubated with 185.5 μg/ml FAC for 18 hours to promote intracellular iron accumulation. According to the batch information provided by the supplier, this corresponds to a concentration of Fe of 32.65 μg/ml. Cells were then thoroughly washed to remove traces of extracellular iron and incubated with 100 μM AA for 6 hours, before exposure to H_2O_2 (Figure 4.9). FAC did not raise DNA damage above control levels by itself or when cells were subsequently incubated with AA. In agreement with what was described above, AA pre-incubation significantly increased the levels of DNA damage caused by H_2O_2. FAC on its own failed to affect H_2O_2-induced DNA damage ($P > 0.05$), but it significantly augmented the exacerbating effect of AA ($P < 0.005$). These data support the possibility that AA exacerbates H_2O_2-induced DNA damage by promoting intracellular Fenton reactions.

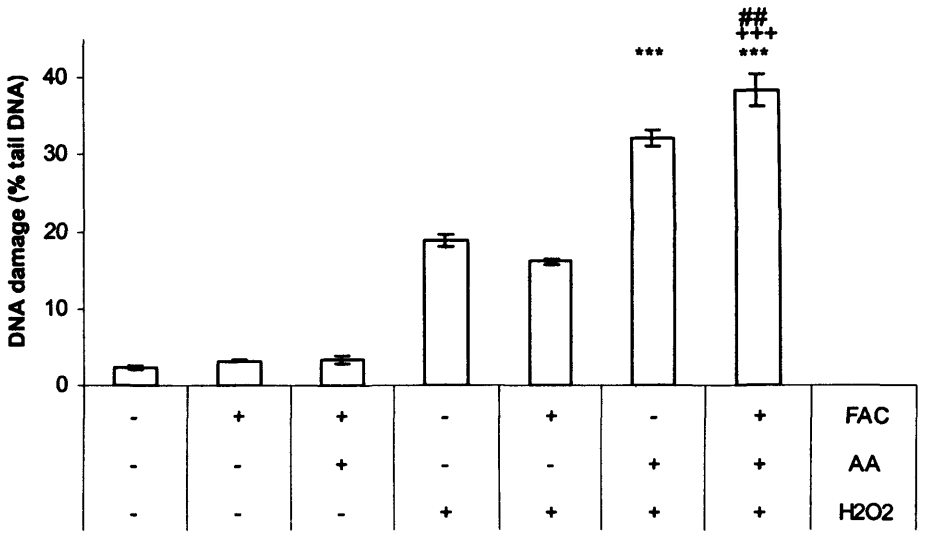


Figure 4.9. Effect of intracellular iron on H_2O_2-induced DNA damage. Cells were incubated with 32.65 μg/ml Fe supplied as FAC for 18 hours. Cells were then thoroughly washed to remove traces of extracellular iron and incubated with 100 μM AA for 6 hours, before exposure to 50 μM H_2O_2. DNA damage was determined with the alkaline comet assay. Results are the mean ± S.D. from 3 separate determinations. ##, $P < 0.005$ *versus* AA pre-incubated, H_2O_2-treated cells; ***, $P < 0.001$ *versus* H_2O_2 treatment without pre-incubation; +++, $P < 0.001$ *versus* FAC pre-incubated, H_2O_2-treated cells.

To further investigate the effects of iron supplementation on H_2O_2-induced DNA damage, experiments were performed in serum-free MEM, which avoids the contribution of serum-derived iron and transferrin. In these experiments, HDFs were grown to confluence, washed thoroughly and incubated in serum-free MEM for 1 hour, during which any serum transferrin that may be bound to or cycling through the cells is secreted into the medium (Oshiro *et al.* 1993). Following a wash with PBS to remove the *apo*-transferrin, cells were incubated with human iron-replete transferrin (*holo*-transferrin, *holo*-transferrin) (10 μg/ml) or FAC (0, 1, 5, 25 or 125 μg/ml) in serum-free MEM with 1 % BSA.

Preliminary experiments showed that H_2O_2 induces higher levels of DNA damage when cells are incubated in serum-free MEM, so a lower concentration of H_2O_2 (20 μM) was used here. FAC increased it in a dose-dependent manner (Figure 4.10A). The effect of FAC on H_2O_2-induced DNA damage was highly significant at 25 or 125 μg/ml. This is in contrast with the effect of FAC on H_2O_2-induced DNA damage in the presence of 10 % FBS (see Figure 4.9), and may possibly be explained by the fact that, in the absence of serum chelators (*e.g.* transferrin), NTBI may enter cells through non-specific transporters and increase the intracellular LIP (Richardson and Baker 1992; Oshiro *et al.* 1993; Papanikolaou and Pantopoulos 2005). In contrast to FAC, iron supplied as *holo*-transferrin did not affect H_2O_2-induced DNA damage ($P > 0.05$) (Figure 4.10B). AA nearly doubled the levels of H_2O_2-induced DNA damage (Figure 4.10B). Notably, addition of extracellular iron either as *holo*-transferrin (10 μg/ml) or FAC (25 μg/ml) significantly reduced the exacerbating effect of AA on H_2O_2-induced DNA damage ($P < 0.05$ and $P < 0.001$, respectively). One possible explanation for this occurrence is that the presence of iron in the medium has caused the oxidation and irreversible loss of AA, with a concomitant reduction of its intracellular accumulation. In this respect, it is worth noting that in the experiments reported in Figure 4.9 HDFs were sequentially incubated with FAC and AA, so AA would only be able to reduce intracellular iron. Here, on the other hand, HDFs were simultaneously incubated with both agents, hence FAC would promote AA oxidation in the medium. The oxidation of AA would, in turn, be favoured by the absence of transferrin in the medium. These observations further confirm that the effect of AA on H_2O_2-induced DNA damage cannot be ascribed to the presence of free iron in cell media.

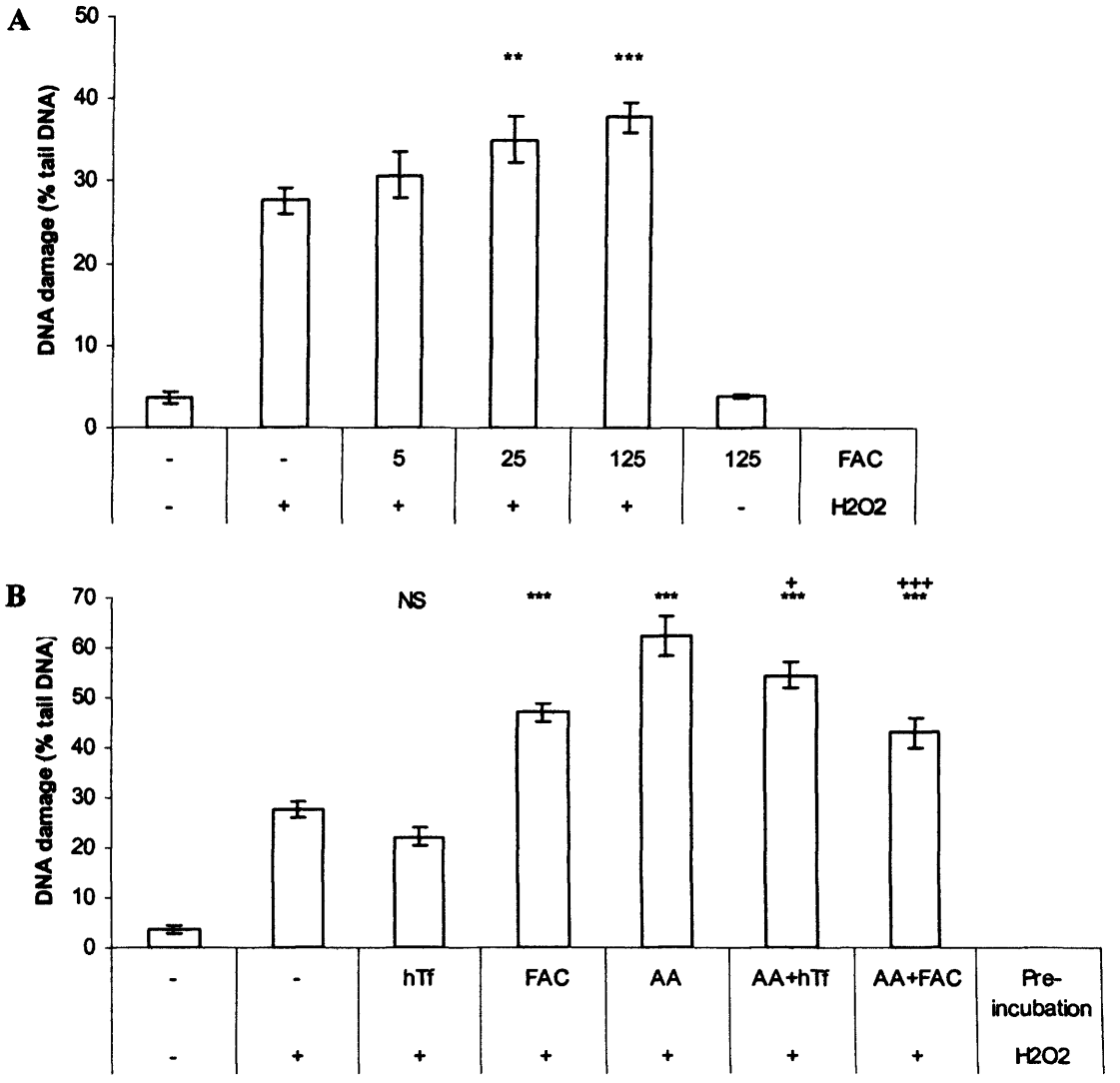


Figure 4.10. Effects of iron and AA on H_2O_2-induced DNA damage in HDFs incubated in serum-free MEM. A, HDFs were thoroughly washed and incubated in serum-free MEM for 1 hour. Following a wash to remove any *apo*-transferrin secreted into the medium, cells were incubated with FAC (0, 5, 25 or 125 μg/ml) in serum-free MEM with 1 % BSA for 6 hours. HDFs were then washed 3 times with PBS and incubated with 20 μM H_2O_2 in serum-free MEM on ice, in the dark for 10 minutes. Results are the mean ± S.D. from 3 separate determinations. **, $P < 0.005$ *versus* H_2O_2 treatment without pre-incubation; ***, $P < 0.001$ *versus* H_2O_2 treatment without pre-incubation. B, HDFs were pre-incubated in serum-free MEM as above and then incubated with either 10 μg/ml human *holo*-transferrin, 25 μg/ml FAC, 100 μM AA or AA in the presence of *holo*-transferrin or FAC in serum-free MEM with 1 % BSA at 37 °C for 6 hours. Cells were then washed and incubated with H_2O_2 as indicated above. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* H_2O_2 treatment without pre-incubation; NS, not significant *versus* H_2O_2 treatment without pre-incubation; +, $P < 0.05$ *versus* AA pre-incubated, H_2O_2-treated cells; +++, $P < 0.001$ *versus* AA pre-incubated, H_2O_2-treated cells.

4.2.3. Effects of vitamin C on radiation-induced DNA damage

The effect of vitamin C pre-incubation on oxidative DNA damage induced by radiation, which is not thought to require intracellular catalytic iron (Ward 1988), was also investigated. Nearly confluent HDFs were pre-incubated with 100 μM AA or AA2P for 12 hours before exposure to 15 Gy X-rays. As expected, radiation caused a significant increase in tail formation (Figure 4.11). Cells that were pre-incubated with AA or AA2P before exposure to radiation had similar levels of DNA damage to their non pre-incubated counterparts ($P > 0.05$). As expected, radiation damaged DNA with no requirement for the presence of unchelated iron, since damage was not prevented when cells were pre-incubated with a concentration of DFO that efficiently abolished H_2O_2-induced DNA damage. In fact, pre-incubation with DFO caused a small but significant ($P < 0.05$) increase in radiation-induced tail formation. These observations further support the view that AA promotes intracellular ROS-mediated, iron-dependent oxidative reactions.

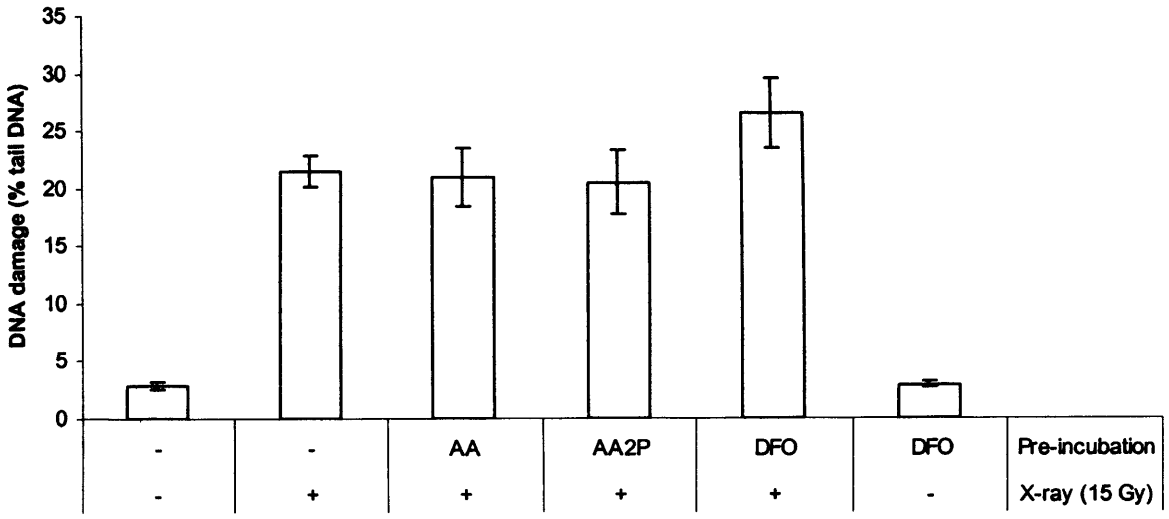


Figure 4.11. Effect of vitamin C pre-incubation on radiation-induced DNA damage. HDFs were incubated with 100 μM AA or AA2P, 300 μM DFO or medium alone for 12 hours. Cells were subsequently exposed to 15 Gy X-ray on ice. Control cells were left on ice for the same period of time. Results are the mean ± S.D. from 6-9 separate determinations.

Table 4.1. Effect of AA and iron supplementation or chelation on the viability of HDFs

	Control	*AA (100 μM)*	*AA2P (100 μM)*	*DFO (300 μM)*	*FAC (185.5 μg/ml)*
% Viability	98.1 ± 0.9	95.8 ± 1.0	96.8 ± 1.8	98.5 ± 0.9	98.3 ± 1.2

Results are the mean ± S.D. from 6-9 separate determinations.

4.2.4. Effects of vitamin C on H_2O_2-induced cytotoxicity

The viability of GM5399 HDFs incubated with AA (100 μM), AA2P (100 μM), DFO (300 μM), FAC (185.5 μg/ml) or growth medium alone (control) for 24 hours was assessed by the trypan blue exclusion method. In this simple method, cells are briefly incubated with a trypan blue solution. Whilst viable cells are able to exclude the dye, cells that have their membrane integrity compromised accumulate it and become blue stained. In all cases, cell viability was higher than 95 %, indicating that none of the treatments was cytotoxic during that period (Table 4.1).

To investigate the effect of AA on H_2O_2-induced cytotoxicity, HDFs were pre-incubated with AA for a certain period of time before exposure to lethal or sub-lethal doses of H_2O_2 at 37 °C. In the current experimental conditions, a sub-lethal treatment consisted of 100 μM H_2O_2 for 1 hour and a lethal treatment was achieved with 200 μM H_2O_2 for 1 hour. HDFs exposed to a sub-lethal dose of H_2O_2 (100 μM for 1 hour) remain viable but enter a senescent-like cell growth arrest (Chen and Ames 1994). This was evident in the present work by the fact that when HDFs were treated just below confluence, H_2O_2-treated HDFs, unlike control cells, did not reach confluence within 24-48 hours in culture. Moreover, an approximately 5-fold increase in the expression of p21 and GADD45α mRNAs was observed as early as 6 hours post-H_2O_2 (Figure 4.12A) and a significant G_0/G_1 cell cycle arrest was evident at 24 and 48 hours post sub-lethal H_2O_2 treatment (Figure 4.12B). Despite this, H_2O_2-treated HDFs remained viable during a long-term incubation period (7.6 % dead cells; $P > 0.05$ *versus* control cells) (Figure 4.13). However, pre-incubation with 100 μM AA for 4 hours caused a significant increase in cell death at 6 days post-H_2O_2 (30 %; $P < 0.001$ *versus* H_2O_2-treated cells without AA pre-incubation), as judged by the cell's inability to exclude the fluorescent dye PI and by a reduction in cell size. Notably, enhanced cell death was prevented when HDFs were incubated with the iron chelator DFO for 14 hours before the 4-hour incubation with AA (6.8 %; $P > 0.05$ *versus* control cells).

When HDFs were exposed to a lethal dose of H_2O_2 (200 μM for 1 hour), approximately 50 % of the cells were killed at 20-24 hours post-H_2O_2 (Figure 4.14).

Notably, cell death was significantly increased in cells pre-incubated with 100 μM AA for 4 hours (82.5 %; $P < 0.001$ *versus* H_2O_2-treated cells without AA pre-incubation). Again, H_2O_2-induced cell death was abolished when cells were loaded with the iron-specific chelating agent DFO for 14 hours ($P > 0.05$ *versus* untreated cells).

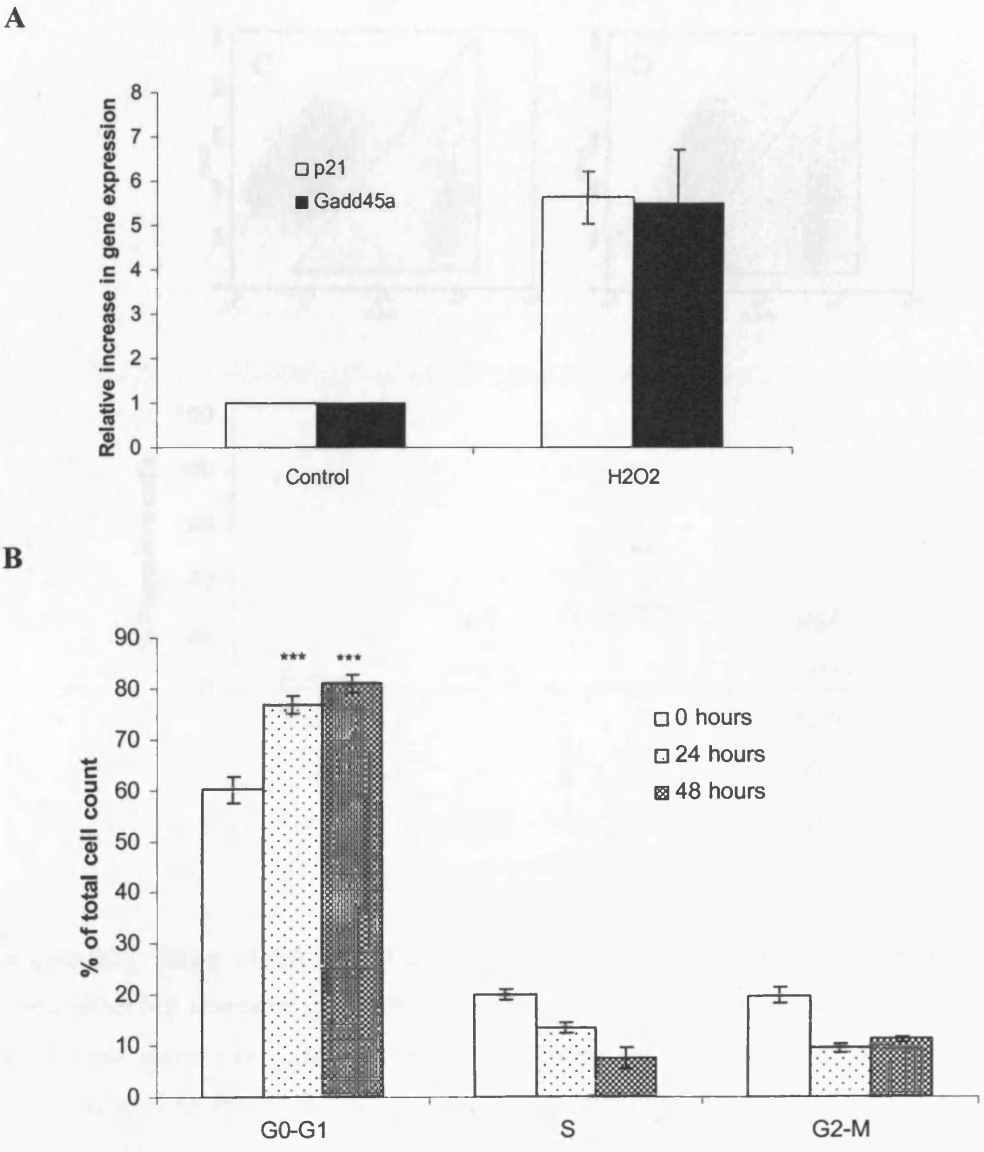


Figure 4.12. Induction of cell cycle arrest by sub-lethal H_2O_2 in HDFs. Cells were exposed to 100 μM H_2O_2 for 1 hour and subsequently incubated in complete growth medium for a certain period of time. A, Expression of p21 and Gadd45α mRNAs at 6 hours post-H_2O_2 was determined by real-time quantitative RT-PCR. Results are the mean ± S.D. from 3 separate determinations. B, Cell cycle distribution was determined at 0, 24 and 48 hours post-H_2O_2. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* 0 hours.

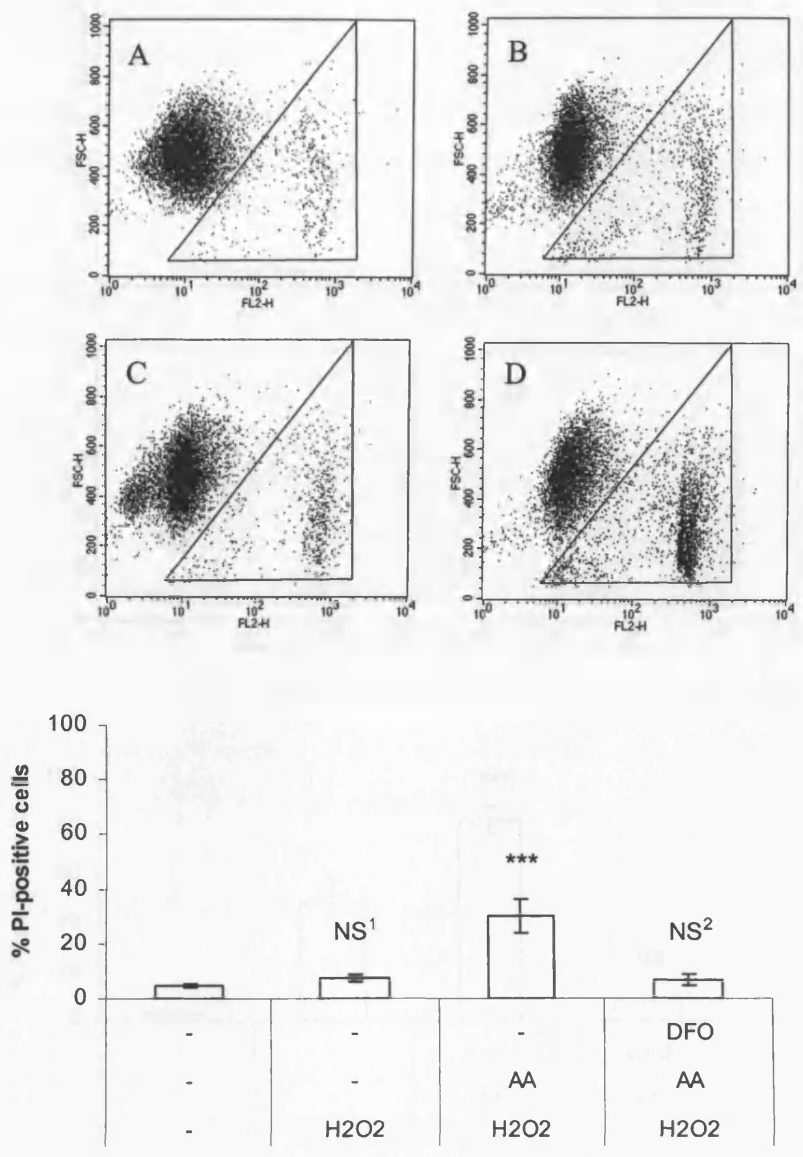


Figure 4.13. Effect of AA on cell death induced by a sub-lethal H_2O_2 challenge in HDFs. Cells were either left untreated (panel A) or exposed to 100 μM H_2O_2 in low-serum medium at 37 °C for 1 hour (panels B, C and D). Some cells were pre-incubated with AA for 4 hours before exposure to H_2O_2 (panel D). Some cells were loaded with 300 μM DFO for 14 hours before the 4-hour incubation with AA (panel C). Cells were washed 3 times with PBS between treatments. Cell viability was estimated at 6 days post-H_2O_2. Dead cells were identified by their inability to exclude the fluorescent dye PI and by a reduction in cell size. Results are the mean ± S.D. from 4 separate determinations. ***, $P < 0.001$ *versus* H_2O_2-treated cells without AA or DFO pre-incubation; NS^1, $P > 0.05$ *versus* control cells; NS^2, $P > 0.05$ *versus* H_2O_2-treated cells without AA or DFO pre-incubation.

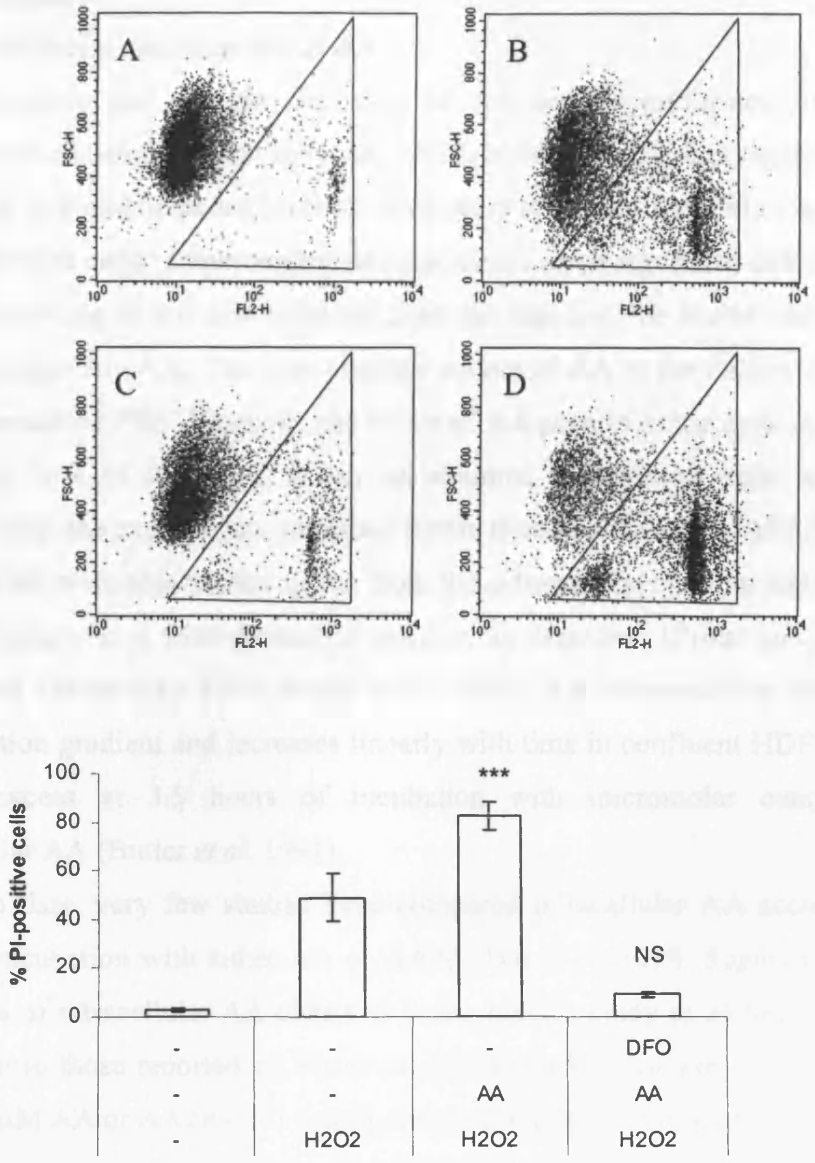


Figure 4.14. Effect of AA on cell death induced by a lethal H_2O_2 challenge in HDFs. Cells were either left untreated (panel A) or exposed to 200 μM H_2O_2 in low-serum medium at 37 °C for 1 hour (panels B, C and D). Some cells were pre-incubated with AA for 4 hours before exposure to H_2O_2 (panel D). Some cells were incubated with 300 μM DFO for 14 hours before the 4-hour incubation with AA (panel C). Cells were washed 3 times with PBS between treatments. Cell viability was estimated at 20-24 hours post-H_2O_2. Dead cells were identified by their inability to exclude the fluorescent dye PI and by a reduction in cell size. Results are the mean ± S.D. from 4-11 separate determinations. ***, $P < 0.001$ *versus* H_2O_2-treated cells without AA pre-incubation; NS, $P > 0.05$ *versus* control cells.

4.3. Discussion

4.3.1. Intracellular accumulation of AA

Sensitive and specific detection of AA can be performed by HPLC with electrochemical detection (Levine *et al.* 1999). In this assay, AA is separated from other substances in a mobile phase by chromatography and, once separation is achieved, AA is detected with either amperometric or coulometric electrochemical detectors.

According to the data obtained from the supplier, the MEM used in this study did not contain any AA. The only possible source of AA in the culture medium would then be the added FBS. However, the levels of AA present in the cells at baseline were below the limit of detection. It can be assumed that control cells were scorbutic. Consequently, the experiments described herein illustrate the effects of AA repletion.

HDFs were able to take up AA from the extracellular medium and to concentrate it intracellularly in a time-dependent manner, as described (Priest and Bublitz 1967; Blanck and Peterkofsky 1975; Butler *et al.* 1991). AA accumulation occurs against a concentration gradient and increases linearly with time in confluent HDFs, leading to a 15-fold excess at 3.5 hours of incubation with micromolar concentrations of extracellular AA (Butler *et al.* 1991).

To date, very few studies have compared intracellular AA accumulation as a result of incubation with either AA or AA2P (Wu *et al.* 1998; Sugimoto *et al.* 2006). The levels of intracellular AA observed in the present study at 24 hours of incubation are similar to those reported by Wu *et al.* (1998) for human skin fibroblasts incubated with 100 μM AA or AA2P (6.41 ± 0.41 and 6.96 ± 1.20 nmol/mg protein, respectively).

4.3.2. Effects of vitamin C on oxidative DNA damage

Pre-incubation of neuroblastoma cells with AA (1 mM for 30 minutes) was reported to increase the DNA strand breakage caused by H_2O_2 (Bruchelt *et al.* 1991), but the statistical significance of this effect was not shown. On the other hand, pre-incubation of Raji lymphoblastoid cells with AA (60 μM) had no significant effect on H_2O_2-induced DNA damage measured with the comet assay (Sweetman *et al.* 1997). In the present study, HDFs were incubated with physiological to pharmacological concentrations of AA (20-500 μM) before exposure to an oxidant insult (H_2O_2 or radiation) and DNA damage was measured using the alkaline comet assay. The version of the comet assay used in this study is based on alkaline unwinding of DNA, allowing the detection of DSBs, SSBs and DNA breaks associated with alkali-labile AP sites,

excision repair sites and strand discontinuities at transcription forks (Singh *et al.* 1988), here collectively referred to as DNA damage (or tail DNA). The percentage of tail DNA relates linearly to the amount of DNA breaks up to about 80 % DNA in tail (Collins 2000), so it can be concluded that the DNA damage values observed herein are well within the linear range of the assay.

AA *per se* did not cause DNA damage but it significantly exacerbated the damaging effects of H_2O_2 in a dose-dependent manner. The exacerbating effect of AA was significant even when low, physiologically relevant extracellular concentrations of AA were employed (20-100 μM) and was linearly related with the intracellular levels of AA, suggesting that the effect of vitamin C on H_2O_2-induced DNA damage may be explained by the intracellular accumulation of AA. The effect was elicited by both AA and the stable vitamin C derivative AA2P, which indicates that it is independent from AA auto-oxidation in the extracellular medium.

The kinetics of DNA strand breakage repair in H_2O_2-treated cells includes a fast and a slow component (Collins and Horvathova 2001). Accordingly, HDFs repaired most of the damage within the first 30 minutes post-exposure to H_2O_2, whereas the remaining damage was nearly all repaired within 60 minutes of incubation, as described for low-passage number HDFs (Wolf *et al.* 2002) and HeLa cells (Duthie and Collins 1997; Potter *et al.* 2002). Due to the very fast repair kinetics of DNA SSBs, some DNA damage is expected to be repaired during cell trypsinisation and slide preparation prior to the comet assay. To minimise DNA repair during sample manipulation, cells were kept on ice and centrifuged at 4 °C, and the periods when the temperature was brought to 37 °C were kept to a minimum (no more than 2 minutes to harvest cells in trypsin-EDTA solution and less than a minute to embed them in LMP agarose). It could be speculated that the differential levels of DNA damage observed in control and AA pre-incubated cells following exposure to H_2O_2 could be due to an effect of the vitamin on the ability of cells to repair DNA damage during the cell processing steps for the comet assay. In fact, Duthie and Collins (1997) have suggested that the susceptibility of different human cell lines to H_2O_2 may be correlated to differences in the cellular capacity to repair DNA strand breakage. In the present study, however, H_2O_2-induced DNA damage was repaired with the same kinetics in control and AA-loaded cells, indicating that DNA lesions measured by the comet assay in both cases are of the same nature and that AA pre-treatment does not affect the cellular repair capacity. So other factors must account for the exacerbating effect of AA on H_2O_2-induced DNA damage.

H_2O_2 can be generated *in vivo* from dismutation of superoxide anion or from several oxidase enzymes. It is poorly reactive and readily diffuses through the cell membrane. Despite its poor reactivity, H_2O_2 can be cytotoxic at high concentrations and hence cells possess specific enzymes to remove it, namely CAT and peroxidases. It is believed that H_2O_2 does not damage cells alone, but rather by reacting with transition metals inside the cells to form much more damaging species such as the hydroxyl radical (Halliwell and Gutteridge 1999). Accordingly, there is evidence that H_2O_2-induced DNA strand breakage requires the presence of intracellular catalytic iron (Schraufstatter *et al.* 1988; Barbouti *et al.* 2001). This is supported by the fact that H_2O_2-induced DNA damage in control and AA-loaded HDFs was completely abolished when cells were pre-incubated with DFO, which is a potent and specific ferric iron chelator (Keberle 1964). To ensure that the effect of AA on H_2O_2-induced DNA damage was dependent on intracellular iron, rather than iron present in the extracellular medium, the AA pre-incubation was performed in the presence of an excess of *apo*-transferrin. The exacerbating effect of AA on H_2O_2-induced DNA damage was not affected by the addition of exogenous *apo*-transferrin to the medium.

Some experiments were performed in serum-free MEM to avoid the contribution of serum-derived iron and transferrin. HDFs were also pre-incubated in serum-free MEM for 1 hour in order to remove serum transferrin that may be bound to or cycling through the cells. The purpose was to investigate whether the effects of AA could be attributed to the presence of trace levels of contaminant iron in the cell medium. In addition, experiments would show whether AA was able to increase intracellular labile iron by promoting iron uptake and/or release by the specific (*holo*-transferrin) or the non-specific (FAC) mechanisms. Results showed that the addition of extracellular iron as either *holo*-transferrin or FAC significantly reduced, rather than enhanced, the exacerbating effect of AA on H_2O_2-induced DNA damage in serum-free conditions. A possible explanation for this occurrence is that the presence of iron in the medium caused the oxidation and irreversible loss of AA, with a concomitant reduction of its intracellular accumulation. This observation has physiological relevance as individuals with iron overload are known to have low levels of AA in the plasma and as the presence of an iron catalyst accelerates AA oxidation in plasma (Young *et al.* 1994; Livrea *et al.* 1996). Furthermore, these observations confirm that the effect of AA on H_2O_2-induced DNA damage described above cannot be attributed to the presence of free iron in cell media.

As the DNA damaging effect of H_2O_2 was iron-dependent, it is possible that AA could augment this effect by expanding the intracellular catalytic iron pool and/or promoting intracellular Fenton chemistry. To assess this, experiments were conducted to ascertain whether intracellular iron accumulation could further augment the exacerbating effect of AA on H_2O_2-induced DNA damage. FAC can be added to cell cultures in order to increase the cellular iron content (Hoepken *et al.* 2004). So, HDFs were sequentially incubated with FAC and AA, before exposure to H_2O_2. FAC significantly augmented the exacerbating effect of AA, which supports the possibility that AA exacerbates H_2O_2-induced DNA damage by promoting intracellular Fenton reactions.

The effect of AA pre-incubation on oxidative DNA damage induced by X-ray was also investigated. The dynamic range for DNA strand break formation with X-ray is 0-8 Gy (Collins 2000). In the present study, however, HDFs were irradiated with a higher radiation dose (15 Gy) and the observed levels of DNA damage were still within the dynamic range of the assay. This can be explained by the fact that, whilst other authors have irradiated cells already embedded in agarose and immediately lysed them, in the present study cells were trypsinised and embedded in agarose after irradiation, which would allow for some DNA repair to occur (McKeown *et al.* 2003). As discussed above, this is a consequence of the very fast kinetics of DNA SSB repair. For example, human lymphocytes repair approximately half of the X-ray induced DNA damage within the first 15 minutes after exposure (Singh *et al.* 1988).

Ionising radiation causes changes in the cells or tissues through which it passes by depositing energy and causing ionisation and excitation of the molecules with which it interacts. Damage to DNA occurs either by direct energy deposition in the DNA molecule or through indirect attack on the DNA molecule of reactive species generated by ionisations in other molecules. The hydroxyl radicals generated from water radiolysis are the main source of this indirect effect (Ward 1988). As expected, the ability of X-rays to damage DNA did not require the presence of intracellular catalytic iron, since it was not prevented by pre-incubation with a concentration of DFO that efficiently abolished H_2O_2-induced DNA damage. In these experiments, HDFs were pre-incubated with AA or AA2P in the same way as for the H_2O_2 experiments, before exposure to X-rays. Notably, vitamin C did not affect radiation-induced DNA damage.

The present study showed that AA exacerbates H_2O_2-induced DNA damage detected by the alkaline comet assay. According to Horváthová *et al.* (1998), the

increase in tail DNA immediately following H_2O_2 treatment reflects the formation of true breaks in the DNA, whereas alkali-labile sites represent only a minor fraction. Although the version of the comet assay employed in the present study does not allow the specific measurement of oxidative lesions, DNA damage was generated using a well-known oxidant, H_2O_2, which is known to damage DNA through the formation of hydroxyl radicals in the presence of redox active metals; the DNA damage measured in the current study should therefore correspond to oxidative damage. The specificity of the assay towards oxidative damage could have been increased by using an adaptation of the method. In this modification of the assay, nucleoids are digested with an endonuclease that recognises a particular DNA lesion and creates a strand break (Collins *et al.* 1993). For example, hOGG1 could be used with the purpose of measuring 8-oxodG formation (Smith *et al.* 2006). Nevertheless, the induction of DNA strand breaks by exposure of human cells to H_2O_2 is well correlated with multiple DNA base lesions (5-hydroxyuracil, 4,6-diamino-5-formamidopyrimidine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 8-hydroxyadenine and 8-hydroxyguanine) (Spencer *et al.* 1995), so AA is expected to enhance the formation of these lesions too. In fact, during the write-up of this work, other authors have reported an enhancing effect of AA on another H_2O_2-induced DNA lesion (Riviere *et al.* 2006). A 30-minute incubation of Jurkat cells with DHA sensitised cells for the formation of 5-hydroxy-2'-deoxycytidine, a product of hydroxyl radical-induced oxidation of 2'-deoxycytidine, by H_2O_2 in a dose-dependent manner. Moreover, the effect was enhanced when cells were pre-incubated with $FeSO_4$ and diminished by pre-incubation with DFO, suggesting that the lesion could be formed by Fenton-type reactions. The work of Riviere *et al.* (2006) with Jurkat cells and the present work with HDFs and human breast epithelial cell lines indicate that AA sensitises different cell types of human origin for H_2O_2-induced DNA damage. Whilst DNA strand breaks and 5-hydroxy-2'-deoxycytidine are not considered mutagenic, it can be speculated that, in the presence of H_2O_2, AA would equally favour the formation of mutagenic lesions such as 8-hydroxyguanine.

The biological relevance of these findings needs to be ascertained. As described in Section 1.4.6, results from studies where white blood cells were collected from patients and subsequently challenged *ex vivo* with an oxidant insult are conflicting. AA supplementation increased resistance to an *ex vivo* H_2O_2 challenge in some cases (Brennan *et al.* 2000) but not in others (Anderson *et al.* 1997; Choi *et al.* 2004). One study showed that AA supplementation significantly increased chromosome aberrations

induced by *ex vivo* BLM challenge of lymphocytes (Anderson *et al.* 1997). Likewise, brief incubation of cultured CHO cells with AA (1-10 μM) increased H_2O_2- and BLM-induced chromosomal aberrations, namely isochromatid breaks (Cozzi *et al.* 1997). The ability of BLM to damage DNA involves its binding to DNA and the production of hydroxyl radical, which depends on the presence of certain transition metal ions (like iron) within the DNA-BLM complex (Evans and Halliwell 1994). In test tube experiments, AA stimulates DNA strand breakage mediated by complexes of ferric iron with BLM (Buettner and Moseley 1992) or NTA (Toyokuni and Sagripanti 1992). The study of Anderson *et al.* (1997), however, suggested that AA could enhance DNA damage by modulating the iron status *in vivo*. Nevertheless, more research is needed to clarify whether the data from the present data and those of Riviere and colleagues (2006) are representative of the *in vivo* situation. Naturally, confounding factors have to be taken into account when comparing the present results with those of *ex vivo* studies. In fact, unlike cultured HDFs, human lymphocytes are not depleted of AA and consequently the efficacy of supplements will depend on the initial AA status of each individual.

4.3.3. Effects of vitamin C on cell viability

The effect of AA on H_2O_2-induced cytotoxicity was investigated by pre-incubating HDFs with AA before exposure to lethal or sub-lethal doses of H_2O_2. Confluent HDFs exposed to sub-lethal oxidative stress remain able to exclude trypan blue and continue to synthesise proteins through a long-term incubation. However, cells enter a senescence-like growth arrest state in which they are unable to synthesise DNA and unable to re-enter the cell cycle in response to a variety of growth factors (Chen and Ames 1994). Consistent with the production of an arrest in the G1 phase of the cell cycle, sub-lethal challenge with H_2O_2 triggers rapid induction of p53 and concomitant increase in the expression of the cyclin-dependent kinase inhibitor p21, which occurs within the first 8 hours post-H_2O_2 and remains elevated for several days. As a consequence, the retinoblastoma remains unphosphorylated (Chen *et al.* 1998; Chen *et al.* 2004; Wang *et al.* 2004), which prevents cells from entering S phase. Stress-induced premature senescence is also characterised by a typically morphology (cell enlargement), increased senescence-associated β–galactosidase activity and transforming growth factor-β1-mediated overexpression of fibronectin, osteonectin, SM22 and apolipoprotein J (Dumont *et al.* 2000; Frippiat *et al.* 2001, 2002). Apoptosis,

on the other hand, can be detected at 7 days post- H_2O_2, but only in approximately 5 % of the cells (Ohshima 2004).

In the present study, a senescence-like growth arrest was induced by incubating HDFs with 100 μM H_2O_2 in low-serum medium for 1 hour and subsequently returning them to complete growth medium. The treatment induced the expression of p21 and GADD45α mRNAs as described elsewhere (Duan *et al.* 2005) and caused a significant G_0/G_1 cell cycle arrest. As expected, cells remained mostly viable (7.6 % dead cells) for up to 6 days post-exposure. HDFs that were pre-incubated with 100 μM AA for 4 hours, however, were significantly sensitised towards H_2O_2 (30 % cell death).

Exposure of HDFs to a lethal oxidative challenge (200 μM H_2O_2 for 1 hour) caused extensive cell death within 24 hours, with dead cells remaining attached and showing marked membrane blebs, presumably corresponding to necrotic cell death, as described (Chen *et al.* 2000). This treatment killed approximately 50 % of the cells. Notably, cell death was significantly increased in cells pre-incubated with 100 μM AA for 4 hours (82.5 %).

Cell death following either lethal or sub-lethal H_2O_2 treatment was prevented when HDFs were previously incubated with the iron-specific chelator DFO. The existence of a pool of intracellular ferric iron has been implicated in the cytotoxicity of H_2O_2. Indeed, the cytotoxicity of H_2O_2 to cultured hepatocytes is prevented by DFO and restored by addition of ferric or ferrous iron to cultures (Starke *et al.* 1985).

Finally, in the comet assay experiments cells were exposed to H_2O_2 on ice to prevent the enzymatic formation or removal of DNA damage. It could be argued that the low temperature could somehow reduce the cellular ability to sequester iron. In such non-physiological conditions, iron would become available for participating in intracellular Fenton reactions that could be augmented by AA. It is useful to notice, however, that the results of the cell viability experiments discussed above mimic those of the DNA damage experiments, and in the former both AA pre-incubation and exposure to H_2O_2 were performed at 37 °C. Still, AA pre-incubation increased H_2O_2-induced cell killing and DFO prevented it, which shows that iron can participate in intracellular Fenton-type reactions at physiological temperature. It is therefore unlikely that the exacerbation of H_2O_2-induced DNA damage reported here would be a consequence of treating cells with H_2O_2 at a low temperature.

4.3.4. Potential mechanisms of AA-mediated oxidative damage

H_2O_2 is generated *in vivo* from dismutation of superoxide anion or from several oxidase enzymes. It is poorly reactive and readily diffuses through the cell membrane. Once inside the cells, H_2O_2 reacts with transition metals to form much more damaging species such as the hydroxyl radical (Halliwell and Gutteridge 1999). Whilst H_2O_2-induced DNA strand breakage formation is inhibited by iron chelators (*e.g.* Schraufstatter *et al.* 1988 and the present study), different mechanisms have been described by which iron is able to enhance oxidative cell damage. A possible mechanism involves diffusion of H_2O_2 into the nucleus, where it would react with metal ions bound to DNA, generating the highly reactive hydroxyl radical (Floyd 1981). Hydroxyl radicals are very short-lived, so they would attack the DNA molecule at the vicinity of the site of formation, causing site-specific damage. In the presence of AA or other physiological reducing agents, the metal catalyst can undergo repeated cycles of reduction and oxidation. A 'multi-hit' mechanism can thus be envisaged to take place close to the metal binding site. This multi-hit mechanism would account for a higher incidence of DSBs than what would be expected in case of random-hits and consequently lead to loss of genetic information (Chevion 1988; Halliwell and Gutteridge 1990).

Alternatively, the oxidant insult may release the metal catalyst from the sites of sequestration within the cell, allowing it to bind DNA (Starke *et al.* 1985). This is supported by studies with the potent and specific ferric iron chelator DFO (Keberle 1964). Due to its hydrophilic nature and its molecular weight of more than 600 Da, DFO does not enter cells by passive diffusion (Lloyd *et al.* 1991). Its lipid solubility is too low for it to diffuse across plasma membranes, so DFO is expected to enter cells by fluid-phase endocytosis and to remain within lysosomal and endosomal vesicles (Lloyd *et al.* 1991). It was thus proposed that the iron associated with ferritin or transferrin within endosomal vesicles is the major source of intracellular iron for chelation by DFO (Octave *et al.* 1983; Laub *et al.* 1985). In fact, *in vitro* experiments showed that DFO can only accelerate iron mobilisation from ferritin at an acidic pH like the one that can be found in lysosomes (Octave *et al.* 1983; Laub *et al.* 1985). As DFO was shown to protect cells from H_2O_2-induced DNA damage (Doulias *et al.* 2003; Kurz *et al.* 2004 and the present study) and cytotoxicity (Starke and Farber 1985; Starke *et al.* 1985 and the present study), it can be hypothesised that these compartments contain the pool of cellular redox-active labile iron required for H_2O_2-induced cell damage. Iron that enters

cells by receptor-mediated uptake of transferrin-TFRC complexes is released from transferrin inside the endosomes as a consequence of the acidic pH. Lysosomes, in turn, are the sites of intracellular degradation of organelles and proteins such as ferritin and metalloproteins. Autophagy of cytosolic ferritin would result in iron mobilisation within lysosomes due to the acidic pH and the activity of proteolytic enzymes. So these compartments are expected to contain a pool of labile iron. Moreover, the acidic conditions and their reducing environment would favour the maintenance of at least a proportion of the iron in the ferrous state. Under oxidative stress conditions, this ferrous iron may cause lipid peroxidation (Schafer and Buettner 2000) and ultimately destabilise the lysosomal membrane. Labile iron can thus be released from lysosomes and diffuse into the nucleus, where it can take part in Fenton reactions. DFO could protect cells against H_2O_2-induced cell damage by keeping lysosomal iron in a non-reactive form and thus preventing endosomal/lysosomal membrane destabilization and concomitant release of low molecular weight iron chelates into the cytosol (Kurz *et al.* 2004; Tenopoulou *et al.* 2005). On the other hand, due to the acidic pH, AA would enhance iron mobilization from ferritin in the lysosome (Tufano *et al.* 1981). During H_2O_2-induced oxidative stress, AA would also favour hydroxyl radical formation from *holo*-transferrin (Aruoma and Halliwell 1987). This would cause endosomal/lysosomal membrane rupture, thus facilitating iron mobilization into the cytosol and nucleus, thereby enhancing oxidative damage.

4.4. Summary

Overall, results showed that the intracellular enrichment of HDFs with AA does not cause DNA strand breakage, but it exacerbates DNA damage induced by sub-lethal H_2O_2 exposure. This is accompanied by an increase of H_2O_2-induced cytotoxicity. Both effects are seemingly related to the ability of AA to increase the levels of intracellular reactive iron and promote Fenton reaction inside the cell. Accordingly, the effect of AA on H_2O_2-induced DNA damage was further enhanced when cells were pre-incubated with iron supplied as FAC and abrogated by intracellular, but not extracellular, iron chelation. AA could increase oxidative DNA damage by promoting iron release from the sites of sequestration within the cell, allowing it to bind DNA, and/or by keeping DNA-bound iron in a reduced state, consequently enhancing hydroxyl radical formation from H_2O_2.

In contrast with H_2O_2-induced DNA damage, strand breakage caused by ionising radiation does not depend on Fenton-type reactions. This could explain the fact that AA, at the same concentration that efficiently exacerbated H_2O_2-induced DNA damage, did not alter levels of radiation-induced DNA damage.

The exacerbating effect of AA on H_2O_2-induced DNA damage was not cell line- and cell type-specific. Notably, it was also dose-dependent and significant even at low, relevant AA doses, which could have implications for human supplementation trials, especially in the cases of iron overload or increased endogenous production of ROS (*e.g.* inflammation).

CHAPTER V

EFFECTS OF VITAMIN C ON IRON METABOLISM IN HUMAN FIBROBLASTS

5.1. Introductory notes on the measurement of intracellular LIP

A number of methods have been developed to measure the cellular 'chelatable' iron and these can be divided into disruptive (*e.g.* analysis of chelatable iron in cell lysates or homogenates by atomic spectroscopy with mass spectroscopy and high-performance liquid chromatography, inductively coupled plasma-mass spectroscopy or electron spin resonance) and non-disruptive methods (*e.g.* radioactive detection, fluorescence spectroscopy) (Petrat *et al.* 2002). The former require sample homogenisation or lysis and the destruction of cell structures may cause the leakage of otherwise firmly bound iron into the chelatable iron pool, thereby leading to an overestimation of the LIP. Among the non-disruptive methods, fluorescence spectroscopic methods offer important advantages: they are highly sensitive, require a small sample, they are less invasive and can be used with living cells (Petrat *et al.* 2002). Fluorescent indicator molecules for the detection of chelatable iron consist of a metal chelating agent coupled with a fluorescent moiety. Whereas the properties of the chelator determine the affinity and specificity of the indicator's response to the metal, the chosen fluorophore (*e.g.* fluorescein) senses the binding of the metal to the chelator and responds with a change in quantum yield (Esposito *et al.* 2002). The most commonly used fluorescent probes in iron detection to date have been calcein (Epsztejn *et al.* 1997) and the phenanthroline analogue PG SK (Petrat *et al.* 1999), both of which respond to iron binding with a rapid quenching of the fluorescence signal. For the detection of intracellular labile iron, cells are usually incubated with the lipophilic (and membrane-permeable) form of the fluorescent indicator (*e.g.* calcein acetoxymethylester, CA-AM, or PG SK diacetate). The chemical structure of PG SK diacetate is depicted in Figure 5.1A. Inside the cells, enzymes hydrolyze the ester bonds of the fluorophore and generate the non-permeable fluorescent indicator, which is retained intracellularly. The detection of iron using these two probes is based on the same principle: chelation of intracellular transit iron by the iron indicator causes a partial quenching of its fluorescence; subsequent dequenching of intracellular fluorescence can be achieved by adding an excess of a strong membrane-permeable chelator like salicylaldehyde isonicotinoylhydrazone (SIH) or BIP (Figure 5.1B), which competes with the metal ion-binding site of the metal indicator leading to a redistribution of cellular chelatable iron from the indicator to the second chelator. The increase in fluorescence produced by the addition

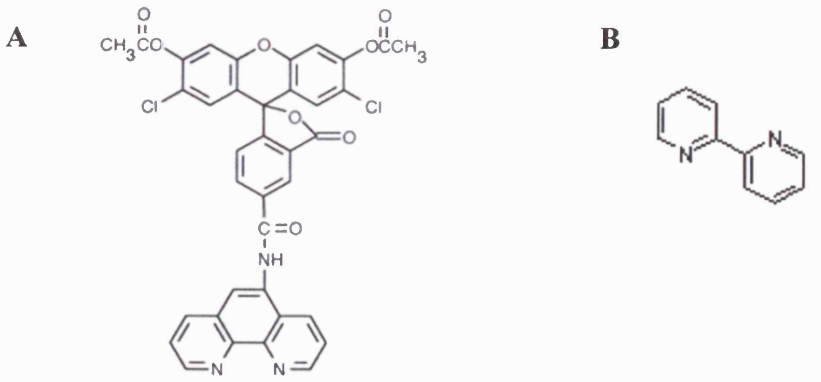


Figure 5.1. Chemical structure of Phen Green SK diacetate (A) and 2,2'- bipyridyl (BIP) (B).

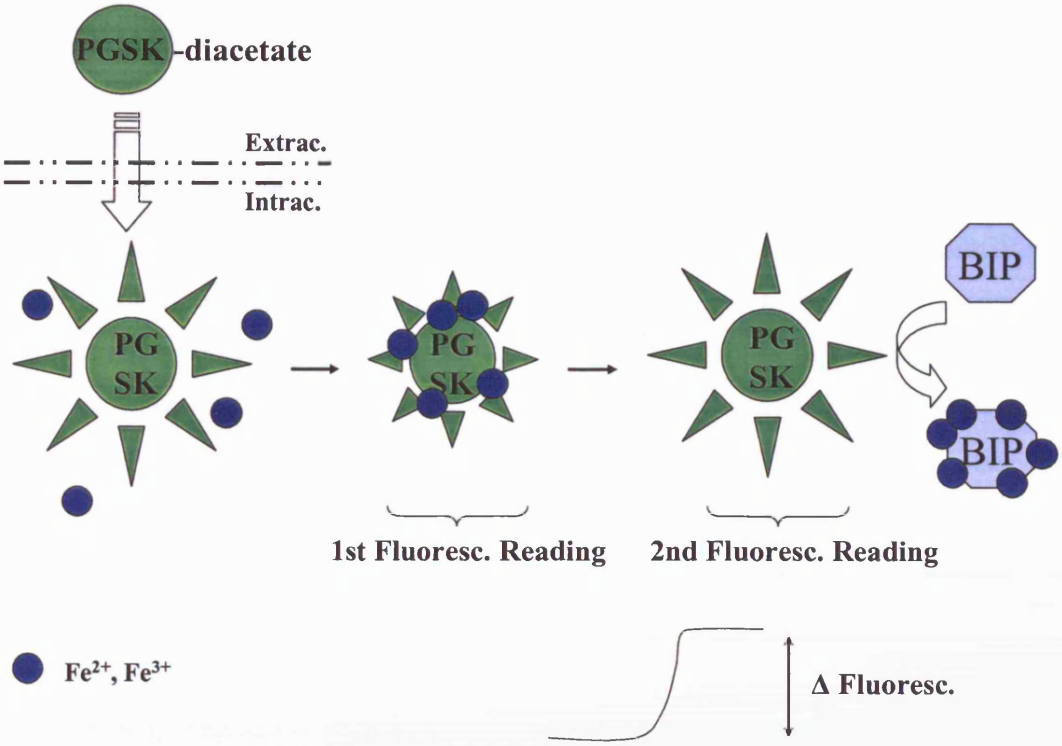


Figure 5.2. Measurement of intracellular chelatable iron with PG SK. Cells are loaded with the membrane-permeable form of the fluorescent indicator (PG SK diacetate). The fluorescent indicator is released enzymatically and accumulates inside cells, where its fluorescence is partially quenched by chelation of intracellular labile iron. PG SK fluorescence is measured before and after the addition of the highly permeable chelator (BIP), which removes all the iron from PG SK and restores its 'initial' fluorescence. The increase in fluorescence produced by the chelator (Δ fluorescence) is proportional to the size of the intracellular LIP.

of the second chelator (Δ fluorescence) is therefore a measure of the intracellular chelatable iron pool that is available to the indicator. The measurement of intracellular chelatable iron with PG SK is depicted in Figure 5.2.

CA-AM has been used to estimate LIP in several cell types, including erythroid and myeloid cells (Epsztejn *et al.* 1997), primary human skin fibroblasts (Pourzand *et al.* 1999; Zhong *et al.* 2004), intestinal epithelial cells (Garate and Nunez 2000), whereas PG SK diacetate has been used to estimate LIP in rat hepatocytes (Petrat *et al.* 2000), guinea pig cochlear epithelium cells (Dehne *et al.* 2001), rat liver endothelial cells (Petrat *et al.* 2001). Typical steady-state levels of intracellular LIP are in the lower micromolar range (1-7 μM). Zhong *et al.* (2004) have analyzed the basal levels of calcein-chelatable iron pool of several human skin fibroblast cell lines and reported that these range between 1.2-2 μM.

5.2. Introductory notes on gene expression analysis by real-time RT-PCR

Real-time RT-PCR is presently one of the most popular quantification methods for gene expression analysis (Bustin 2000). It combines the sensitivity of RT-PCR with the flexibility of fluorescence-based kinetic (or real-time) PCR, allowing the simultaneous measurement of gene expression changes in different samples and for specific genes. The reverse transcription step is necessary to convert the mRNA targets into cDNA, which can serve as a template for subsequent PCR amplification. Two reverse transcriptase enzymes are commonly employed in the RT step, avian myeloblastosis virus reverse transcriptase or Moloney murine leukaemia virus reverse transcriptase and the reaction is primed using specific primers, random hexamers or oligo(dT) primers. The latter two procedures allow the study of different mRNA targets from a sample of RNA. The PCR reaction is usually performed with Taq DNA polymerase in the presence of either a DNA-binding fluorescent dye (SYBR Green) or a fluorescence-labelled oligonucleotide probe specific for the target transcript. The reaction is carried out in a thermal cycler that is capable of real-time fluorescence measurement.

SYBR Green fluoresces only weakly when in solution and in the presence of single-stranded DNA. However, its fluorescence increases by approximately 1000-fold when bound to double-stranded DNA. In a PCR reaction, this occurs at the elongation and extension steps. Hence, fluorescence can be measured at each cycle of the polymerisation

reaction to monitor the amount of amplified DNA, as illustrated in Figure 5.3A. The amplification curves of multiple samples are best looked at by plotting log fluorescence *versus* cycle number (Figure 5.3B). The principle of real-time PCR quantification is that the more template DNA is present at the beginning of the reaction, the fewer number of cycles are required to achieve a certain fluorescence level which is determined to be significantly above the background fluorescence. The PCR cycle at which fluorescence reaches a threshold (Ft) value of ten times the standard deviation of the baseline fluorescence emission is called threshold cycle (Ct). Ct values, which are thus inversely proportional to the log of the initial amount of template (Figure 5.3C), constitute the basis for performing the quantification analysis, as described by Rutledge and Cote (2003). In a PCR reaction, the exponential amplification can be described according to equation 21, where C is the number of cycles, N_C is the number of molecules at cycle C, N_0 is the initial number of molecules and E stands for the amplification efficiency:

$$N_C = N_0 \times (E + 1)^C \qquad [21]$$

Equation 21 can be re-arranged in order to determine the initial number of molecules:

$$N_0 = N_C/(E + 1)^C \qquad [22]$$

Considering that all PCR reactions are compared at the same Ft, *i.e.* when the same number of molecules is reached, then N_C becomes a constant value, as indicated in equation 23, where Ct is the threshold cycle and Nt is the number of molecules at Ft.

$$N_0 = N_t/(E + 1)^{Ct} \qquad [23]$$

Quantification relies on standard curves constructed by amplification of known amounts of target DNA and plotting Ct values against the log template DNA [$Log(N_0)$] (*e.g.* Figure 5.3C). Thus the equation of the standard curve can be derived by taking the logarithm of equation 23, as follows:

$$Log(N_0) = Log(Nt) - Log[(E + 1)^{Ct}] \qquad [24]$$

$$Log(N_0) = Log(Nt) - Log(E + 1) \times Ct \qquad [25]$$

$$Log(N_0) = - Log(E + 1) \times Ct + Log(Nt) \qquad [26]$$

$$Ct = - 1/Log(E+1) \times Log(N_0) + Log(Nt)/Log(E+1) \qquad [27]$$

E and Nt are assumed to be constants, so the equation above is the equation of the line ($y = mx + b$) obtained when plotting $Log(N_0)$ *versus* Ct. In this equation, y is the Ct value, m is the slope of the line, x stands for $Log(N_0)$ and b is the intercept:

$$Ct = slope \times Log(N_0) + intercept \qquad [28]$$

The amplification efficiency can thus be estimated from the slope of the line:

$$Slope = -1/Log(E + 1) \qquad [29]$$

$$E = 10^{1/-Slope} - 1 \qquad [30]$$

At an efficiency of 100 %, one cycle (expressed as Ct) corresponds to a 2-fold change in the amount of target. An ideal slope should be –3.323 for 100 % PCR efficiency. At the same time, the initial template quantity can be estimated from the regression line equation:

$$Log(N_0) = (Ct - intercept)/ slope \qquad [31]$$

$$N = 10^{(Ct - intercept)/slope} \qquad [32]$$

The Ct always occurs during the exponential phase of amplification. Collecting information at this stage is an advantage of real-time PCR over the more traditional endpoint quantitative PCR, where quantification is usually performed by measuring band intensity after agarose gel electrophoresis by means of image analysis software. Whilst endpoint measurements are easily affected by reagent depletion or accumulation of polymerase reaction inhibitors during the 'plateau' phase of the amplification, real-time quantification measures product formation during the exponential phase of the reaction and will thus more accurately relate to the initial template quantity (Bustin 2000).

One possible disadvantage of using the SYBR Green assay is that it is not gene-specific. In fact, SYBR Green shows equal affinity for any double-stranded DNA molecules formed during the amplification reaction, such as primer dimers and non-specific products. The specificity can, however, be improved by performing a dissociation (melting curve) analysis on the PCR product (Figure 5.3D). This is achieved by gradually increasing the temperature above the melting temperature of the amplicon and measuring fluorescence; subsequently, fluorescence (more precisely, the negative first derivate of the raw fluorescence) is plotted as a function of temperature. SYBR Green fluorescence increases markedly when bound to double-stranded DNA, so a steep loss of fluorescence occurs when temperature exceeds the melting temperature of the amplification product. Thus, the observed dissociation peak corresponds to that sudden drop in fluorescence. As the melting temperature depends greatly on amplicon size and nucleotide composition, the amplification of a single product yields a unique dissociation peak in the curve, whereas the presence of non-specific products or primer dimers would generate extra peaks.

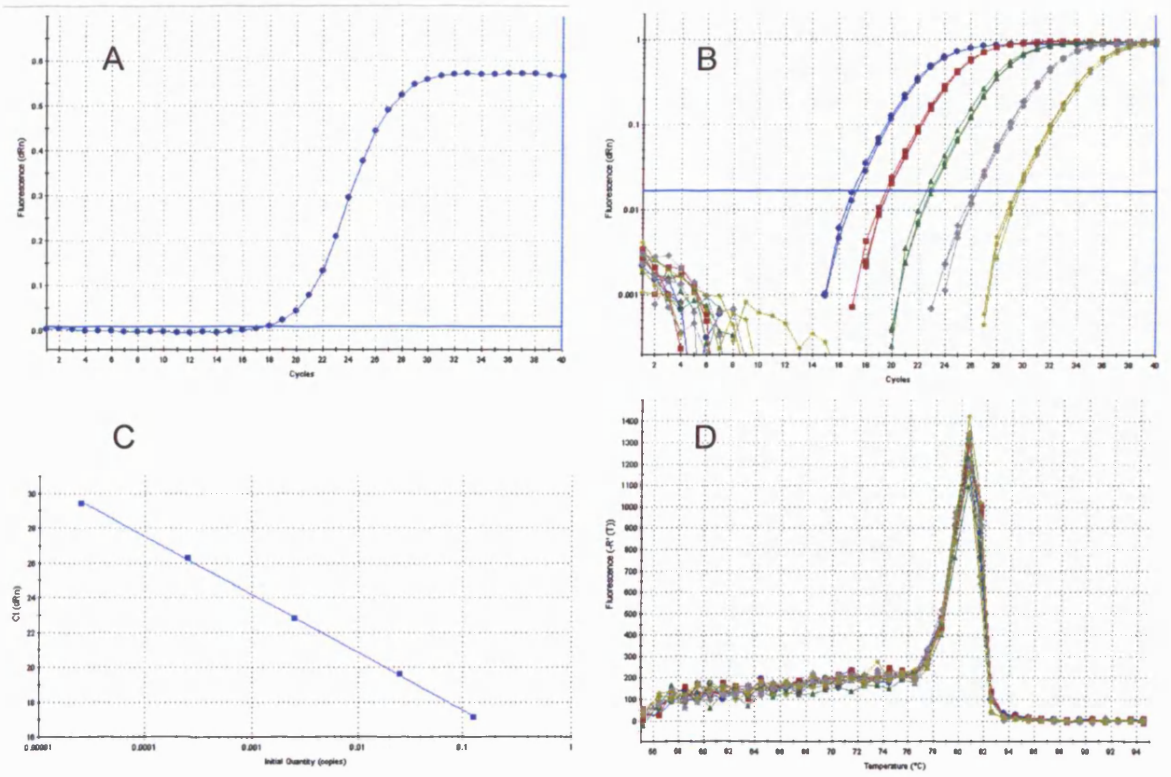


Figure 5.3. Quantitative real-time RT-PCR analysis. A, Real-time detection of PCR amplification products by measurement of the fluorescence emitted by SYBR Green at the end of the annealing and elongation steps of each amplification cycle. SYBR Green fluoresces when bound to double-stranded DNA, so the fluorescent signal increases with increasing amounts of DNA molecules formed. The horizontal blue line is the fluorescence threshold (Ft). B, Amplification curves of multiple PCR products obtained by plotting cycle number *versus* the log of measured fluorescence, in which the linear region represents the exponential phase of amplification. The Ft is the point at which Ct values are determined for all individual samples. C, Standard curve generated by plotting the log of input material (template DNA concentration) *versus* threshold cycle (Ct). The efficiency of the reaction is calculated from the slope of the curve (% PCR efficiency = $100 \times 10^{(1/\text{-slope})} - 1$). The amount of template in the experimental samples is calculated from the standard curve as (observed Ct – y intercept)/slope. D, Dissociation curves of multiple PCR products. The existence of a single melting point indicates the presence of a single amplification product.

Naturally, the accuracy of the quantification can be affected by differences in the amount of starting material, in the reverse transcription and in the PCR reaction. This can

be corrected by amplifying, in the same sample, an endogenous control gene or by normalising the results against T-RNA concentration. Correction for T-RNA, however, relies on an accurate RNA quantification method and does not correct for all possible sources of variation (*e.g.* reverse transcription and PCR inhibitors) (Bustin 2002). Therefore, the expression of a gene of interest is commonly normalised against that of a reference gene. Reference genes represent endogenous gene expression controls, allowing sample normalisation against differences introduced during sample manipulation (*e.g.* differing amounts of input RNA and unequal reverse transcription efficiencies). The choice of reference genes is, however, crucial for the interpretation of gene expression data. An ideal reference gene should be expressed at a similar level as the genes of interest and its expression should be stable among different tissues of an organism, during all stages of development and across all experimental treatments (Bustin 2000). 'Housekeeping genes', *i.e.* genes that are expressed in all nucleated cell types as their function is essential for cell survival, have been widely used as reference genes in gene expression analysis. Traditionally it has been assumed that their expression is very stable. Some of the most commonly used 'housekeeping' genes are β-actin, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), cyclophilin, α- and β-tubulin, ribosomal protein L32 and ribosomal RNA subunits 18S and 28S (Thellin *et al.* 1999). However, recent evidence shows that their expression levels vary considerably in biological samples and therefore their usefulness as endogenous controls has been disputed. Some authors have preferred to use ribosomal RNAs, but these are generated by different transcriptases and expressed at much higher levels than target mRNAs (Bustin 2000). It appears that no single gene can be an optimal endogenous control in all systems, so the selection of a reference gene should ideally be validated for each particular experimental model (Dheda *et al.* 2004).

Relative quantification measures changes in steady-state transcription of a gene. In experiments where expression levels are normalised to a reference gene, standard curves are prepared for the target and the endogenous reference gene. A relative standard curve can be constructed by preparing a serial dilution of a sample of known concentration (*e.g.* Figure 5.3C). Because the sample quantity is divided by the calibrator (*i.e.* control sample) quantity, the unit from the standard curve is dropped out. So, for the purposes of relative quantification, the concentration of the standard can be expressed in arbitrary units (*e.g.*

dilution factor). For each experimental sample, the target and reference quantities are estimated from the respective standard curves. Then the target quantity is divided by the quantity of the reference gene to obtain a normalised expression value. In addition, a calibrator sample can be used as the basis for comparative quantification. This calibrator sample is typically a control or untreated sample that was otherwise processed in a similar way as the experimental samples. For all experimental samples, the normalised expression value for a target gene is divided by the normalised expression value of the calibrator and all quantities are then expressed as an *n*-fold relative to the calibrator sample.

5.3. Specific aims of study

As mentioned in Section 1.3.6, the majority of the intracellular iron is safely bound to storage, functional or transport proteins, but a small pool of cellular iron is loosely bound to low molecular weight organic chelators (labile iron pool, LIP). Mammalian cells regulate iron levels tightly through the levels of proteins involved in iron uptake (TFRC) and storage (ferritin) (see Section 1.3.7). Results presented in Chapter IV proposed that AA could enhance oxidative damage by increasing the levels of intracellular reactive iron. Therefore, the specific aim of this study was to investigate the effects of AA on the levels of intracellular reactive iron and whether AA could concomitantly affect the expression of iron-regulated genes in HDFs. HO-1 is another gene involved in iron metabolism. It is involved in the removal of haem iron but its activity may contribute to a transient increase in transit iron. The effect of AA on HO-1 expression was studied here too.

The effects of AA were compared to those of two well-established modulators of the LIP and of iron-regulated gene expression, DFO and FAC. DFO chelates the intracellular iron pool, leading to a concomitant increase in transferrin-dependent iron uptake and reduction in ferritin synthesis. On the other hand, incubation with FAC increases intracellular iron content and has opposite effects to DFO on iron uptake and storage. Therefore, FAC and DFO are well suited to modulate cellular iron levels (Richardson and Baker 1992).

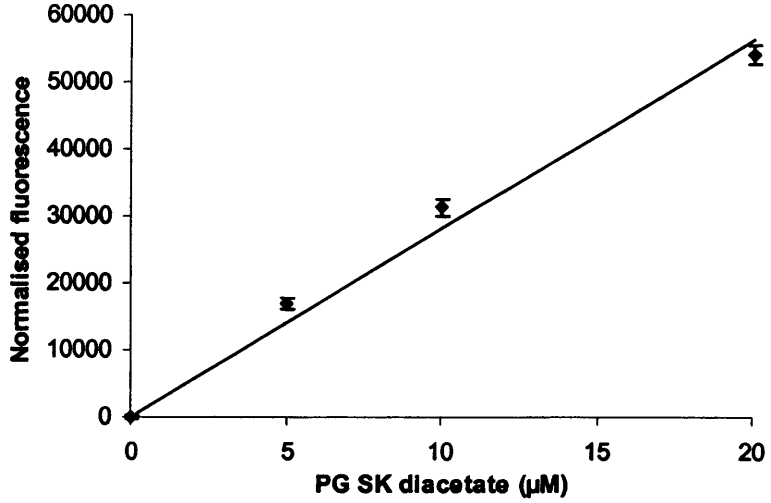
5.4. Results

5.4.1. Effects of vitamin C on the intracellular LIP

The experiments described in Chapter IV have shown that vitamin C enhanced H_2O_2-induced DNA damage and cell death by an iron-dependent process. It is thus possible that vitamin C may promote Fenton-type oxidative damage by increasing the availability of intracellular catalytic iron. The majority of the intracellular iron is safely bound to storage, functional or transport proteins, but a small pool of cellular iron is loosely bound to low molecular weight organic chelators. This LIP is thought to be available for participating in the formation of highly reactive oxidant species (Stohs and Bagchi 1995). Changes in the intracellular LIP of HDFs were measured here using an adaptation of the method of Petrat *et al.* (1999). This method is based on quenching the fluorescence of the transition metal indicator PG SK by intracellular iron and its subsequent dequenching upon addition of a strong membrane-permeable iron chelator, BIP. By performing the assay on cells grown in 96-well plates, it was possible to measure the LIP in multiple samples in parallel.

In preliminary experiments, HDFs were incubated with different concentrations (0-20 μM) of the membrane-permeable form of the fluorescent indicator, PG SK diacetate. Cellular enzymes hydrolyze the ester bonds of the fluorophore and generate the non-permeable fluorescent indicator, which is retained intracellularly. To assess the dose-dependence of the uptake of the fluorescent indicator, fluorescence was measured after the addition of BIP. This should ensure that the fluorescence measured is directly proportional to intracellular concentration of the fluorescent indicator. Results show that HDFs accumulated the metal indicator, PG SK, in a dose-dependent manner, as indicated by a linear increase in fluorescence (Figure 5.4A). Results suggest, however, that some fluorescence saturation may have occurred at the highest dose of PG SK diacetate, so subsequent experiments were performed using 10 μM PG SK diacetate. As expected, HDFs incubated with PG SK diacetate responded to the addition of the iron chelator, BIP, with a clear and significant increase in fluorescence signal (Figure 5.4B), which is thought to be due to the release of iron from PG SK. This indicates that these cells have a detectable pool of labile (or 'chelatable') iron.

A



B

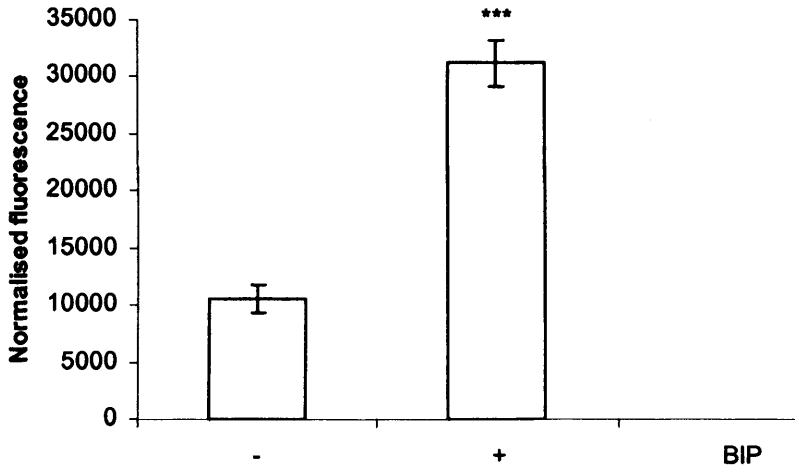


Figure. 5.4. Cellular uptake of Phen Green SK diacetate. A, HDFs were incubated with 0, 5, 10 or 20 μM PG SK diacetate for 10 minutes at 37 °C. Fluorescence was measured after the addition of the permeable chelator BIP, as described in Materials and Methods. Results are the mean ± S.D. of six replicate wells. B, Cells were incubated with 10 μM PG SK diacetate for 10 minutes at 37 °C. Fluorescence was measured just before and 15 minutes after the addition of BIP, as described in Materials and Methods. Results are the mean ± S.D. of six replicate wells. ***, $P < 0.001$ *versus* fluorescence before addition of BIP.

Previous work showed that pre-incubation with DFO (300 μM) efficiently chelates the intracellular LIP of human cells (Breuer *et al.* 1995), whereas incubation with FAC expands the LIP (Hoepken *et al.* 2004). As expected, incubation of HDFs with 300 μM DFO for 12 hours produced a smaller increase in fluorescence signal after addition of BIP (Δ fluorescence) than that observed in control cells, showing significant reduction in the levels of intracellular chelatable iron ($P < 0.001$ *versus* control) (Figure 5.5). Conversely, incubation with 32.65 μg/ml Fe in the form of FAC for 15 hours increased this iron pool significantly ($P < 0.001$ *versus* control). To investigate the effect of vitamin C on the intracellular LIP, HDFs were incubated with either AA or AA2P (100 μM) for 12 hours. Notably, both vitamin C derivatives caused significant increases in intracellular PG SK-chelatable iron, as illustrated by an increase in Δ fluorescence when comparing with untreated cells ($P < 0.001$ *versus* control).

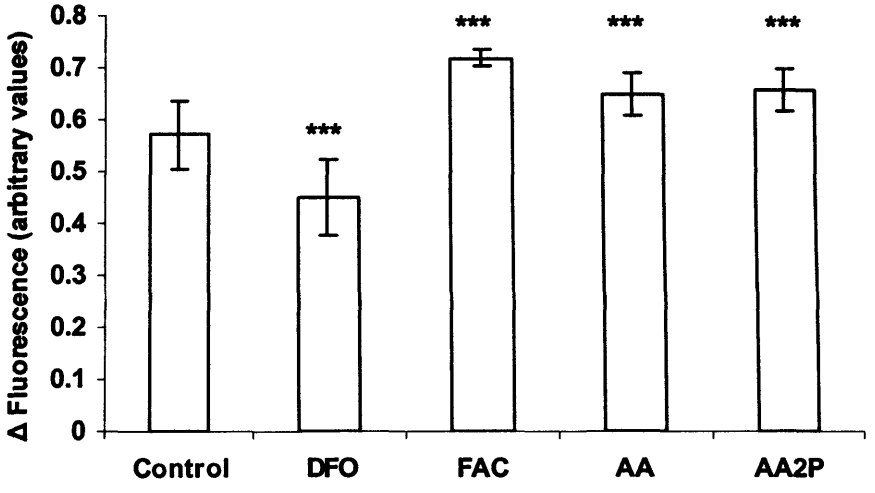


Figure 5.5. Effect of vitamin C on the intracellular chelatable iron pool of HDFs. Cells were incubated in 96-well plates with either 300 μM DFO or 100 μM AA or AA2P for 12 hours, or with 32.65 μg/ml Fe supplied as FAC for 15 hours. Cells were then incubated with 10 μM PG SK diacetate for 10 minutes at 37 °C. Fluorescence was measured just before and 15 minutes after the addition of the permeable chelator BIP, as described in Materials and Methods. The increase in fluorescence produced by the chelator (Δ fluorescence) is proportional to the size of the intracellular LIP. Results are the mean ± S.D. of multiple replicate wells pooled from four independent experiments. ***, $P < 0.001$ *versus* control.

5.4.2. Effects of vitamin C on iron-regulated gene expression

i) TFRC expression

Mammalian cells respond promptly to changes in the intracellular LIP by regulating the levels of proteins involved in iron uptake (TFRC) and storage (ferritin). So, if AA is capable of expanding the cellular LIP, it should also lead to changes in the expression of these two iron-regulated genes. Iron-dependent regulation of TFRC expression is determined by mRNA stability, namely due to the existence of five stem-loop structures containing IREs in the 3'-UTR of the mRNA (Kühn *et al.* 1984; Owen and Kühn 1987; Klausner *et al.* 1993). The binding of IRPs to IREs occurs when iron is insufficient and increases the half-life of TFRC mRNA, whereas excessive iron has the opposite effect. In the present work, the steady-state levels of the TFRC mRNA were measured by real-time quantitative RT-PCR. T-RNA was extracted from cells and its integrity determined by denaturing gel electrophoresis. The RNA samples used in this study were of good quality, as indicated by the presence of distinct bands corresponding to the intact 28S and 18S rRNA subunits and the absence of low molecular mass degradation products (Figure 5.6). Primers were designed for the specific amplification of a short fragment in the 3'-UTR of the TFRC mRNA, as indicated in Appendix III. Primers were also designed for the amplification of transcripts from an endogenous control gene, HPRT1. Amplification conditions were optimised for each set of primers. The absence of amplification product in the no-template controls confirmed that the results obtained were not affected by external DNA contamination, whereas the absence of amplification product in the no-reverse transcriptase (no-RT) controls confirmed that the results obtained represent the amplification of cDNA, rather than of genomic DNA still present in the T-RNA sample after the DNase digestion. A representative amplification plot including no-template and no-RT controls is shown in Figure 5.7.

The amplifications curves, standard curve, dissociation curves corresponding to the optimised amplification conditions for HPRT1 and TFRC are shown in Figures 5.8 and 5.9, respectively. Standard curves generated by linear regression analysis of log standard DNA *versus* Ct were used to calculate reaction efficiency and to estimate target quantity for each individual sample, as described in Sections 2.10.5 and 5.2. R-square values indicating 'goodness of fit' of the regression line ranged between 0.998 and 1.000. The amplification

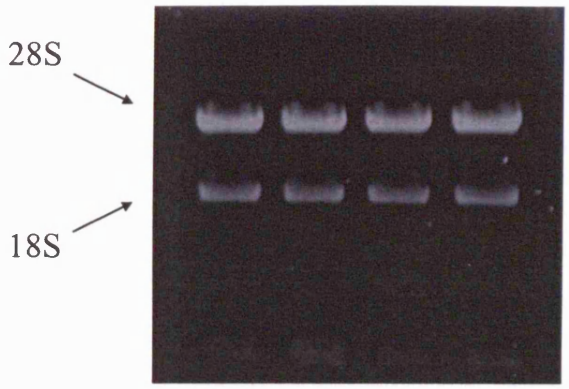


Figure 5.6. Size distribution of T-RNA samples. T-RNA extracted from cells was analysed by denaturing gel electrophoresis as described in Materials and Methods. The example shown is representative of all the samples used in this study.

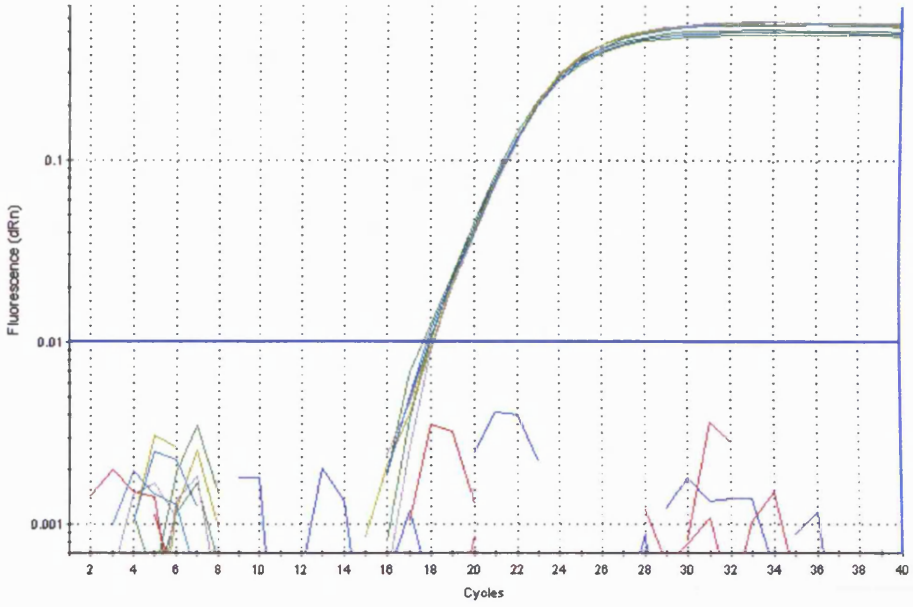


Figure 5.7. Representative real-time RT-PCR amplification curve. Amplification of HPRT1 transcripts in two sets of replicate samples (light and dark green), no-template control (red) and no-RT control (blue) was performed as described under Materials and Methods. The horizontal blue line represents the fluorescence threshold (Ft).

efficiencies of the standards ranged between 90 % and 100 %. The dissociation curves of the amplification products showed a single peak, confirming the specificity of the reaction and the absence of primer dimers. Further confirmation of the reaction specificity was obtained by analysis of the amplification products by gel electrophoresis, which showed a single band with the expected size (Figure 5.10).

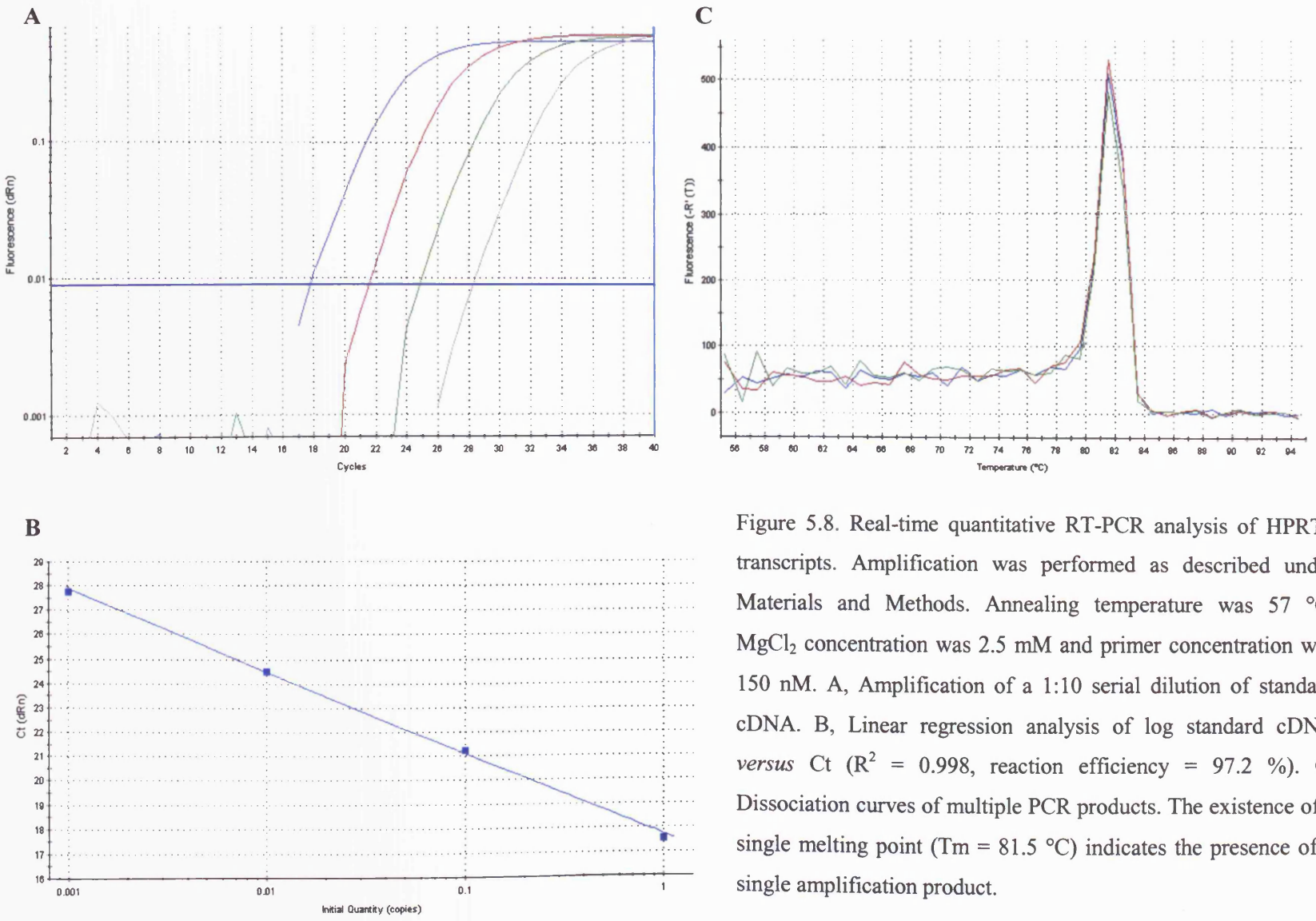


Figure 5.8. Real-time quantitative RT-PCR analysis of HPRT1 transcripts. Amplification was performed as described under Materials and Methods. Annealing temperature was 57 °C, $MgCl_2$ concentration was 2.5 mM and primer concentration was 150 nM. A, Amplification of a 1:10 serial dilution of standard cDNA. B, Linear regression analysis of log standard cDNA *versus* Ct (R^2 = 0.998, reaction efficiency = 97.2 %). C, Dissociation curves of multiple PCR products. The existence of a single melting point (Tm = 81.5 °C) indicates the presence of a single amplification product.

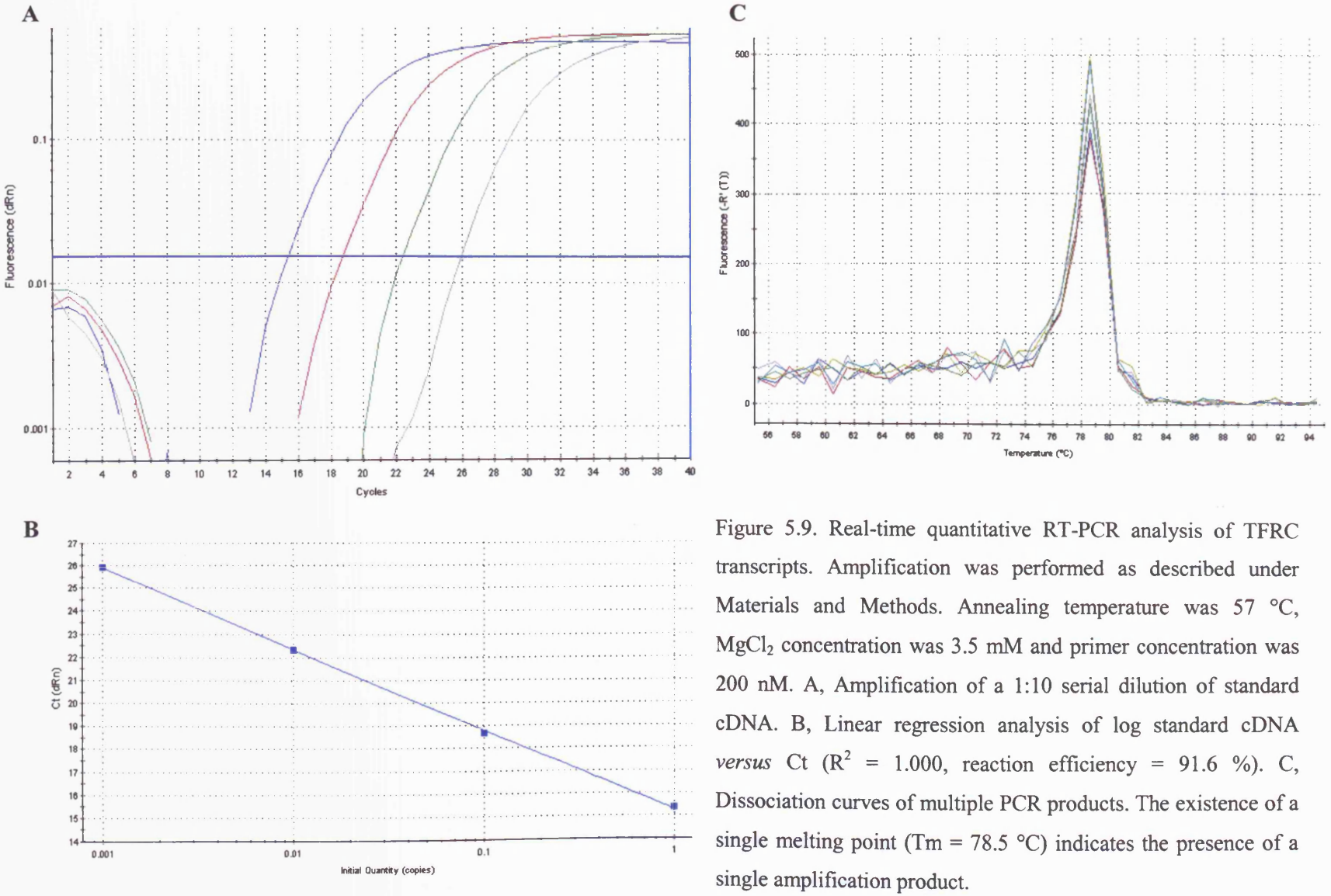


Figure 5.9. Real-time quantitative RT-PCR analysis of TFRC transcripts. Amplification was performed as described under Materials and Methods. Annealing temperature was 57 °C, $MgCl_2$ concentration was 3.5 mM and primer concentration was 200 nM. A, Amplification of a 1:10 serial dilution of standard cDNA. B, Linear regression analysis of log standard cDNA *versus* Ct (R^2 = 1.000, reaction efficiency = 91.6 %). C, Dissociation curves of multiple PCR products. The existence of a single melting point (Tm = 78.5 °C) indicates the presence of a single amplification product.

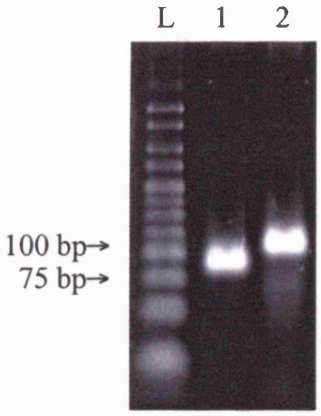


Figure 5.10. Analysis of TFRC and HPRT1 RT-PCR amplification products by gel electrophoresis. An aliquot of PCR product was examined on a 3 % agarose gel. Electrophoresis was run as described in Materials and Methods and the PCR products visualised under UV after EtBr staining. L, DNA HyperLadder V; 1, TFRC; 2, HPRT1.

Relative quantification of the steady-state levels of TFRC mRNA was performed by real-time RT-PCR, using HPRT1 as an endogenous control gene. The effects of iron chelation or loading on TFRC mRNA expression were assessed by incubating nearly confluent GM5399 HDFs with DFO or FAC, respectively. As expected, HDFs responded to iron overload (32.65 μg/ml Fe supplied as FAC for 12 hours) by decreasing the levels of TFRC mRNA substantially (4-fold down-regulation, $P < 0.001$) (Figure 5.11A). Conversely, iron chelation with 300 μM DFO for 12 hours doubled the steady-state levels of TFRC mRNA ($P < 0.001$). This agrees with the current understanding of the regulation of the TFRC expression in HDFs (Ward *et al.* 1982, 1984; Knisely *et al.* 1989). Notably, incubation of HDFs with 100 μM AA or AA2P for 12 hours caused a 2-fold down-regulation of TFRC mRNA ($P < 0.001$; Figure 5.11B). To investigate the time-dependence of TFRC mRNA regulation by vitamin C, HDFs were incubated with 100 μM AA or AA2P or medium alone for 2, 6, 12 and 24 hours. Results show that the effect of vitamin C was time-dependent, with the levels of TFRC mRNA decreasing gradually during the first 6 hours of incubation and then remaining significantly down-regulated until at least 24 hours from the addition of AA or AA2P (Figure 5.11C). The results presented above were obtained with GM5399 cells, but a significant down-regulation of the TFRC mRNA was also observed in nearly confluent GM5659 HDFs incubated with AA or AA2P for 12 hours (Figure 5.12), which suggests that the effect described above is a common response of HDFs to vitamin C.

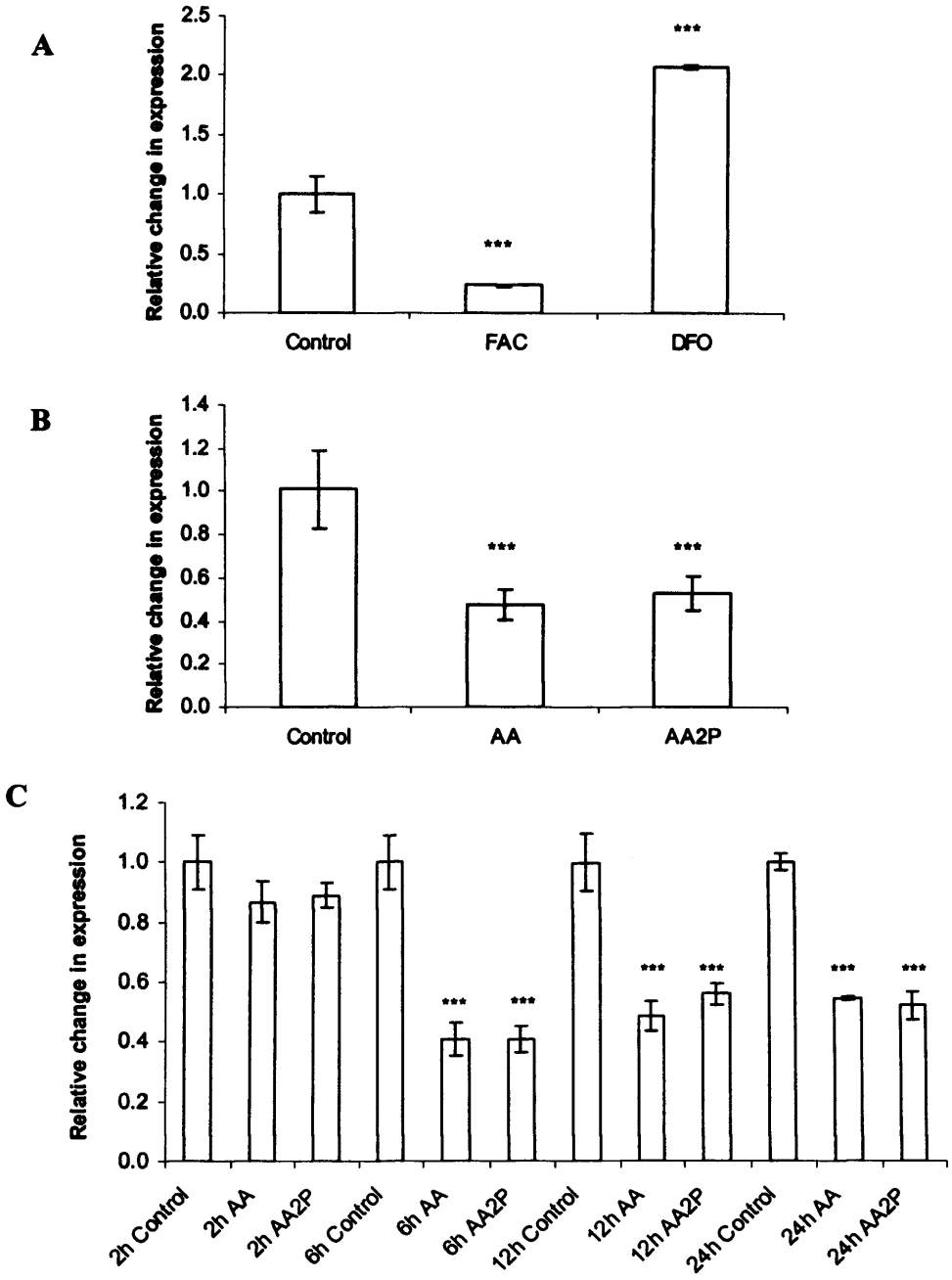


Figure 5.11. Effect of vitamin C on TFRC mRNA expression in GM5399 HDFs. Total RNA was extracted at the end of each treatment and the steady-state levels of TFRC mRNA measured by real-time RT-PCR. A, HDFs were incubated with 32.65 μg/ml Fe (FAC), 300 μM DFO or medium alone for 12 hours. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* control. B, HDFs were incubated with 100 μM AA or AA2P or medium alone for 12 hours. Results are the mean ± S.D. from 6 separate determinations. ***, $P < 0.001$ *versus* control. C, HDFs were incubated with 100 μM AA or AA2P or medium alone for 2, 6, 12 and 24 hours. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* the time-matched control.

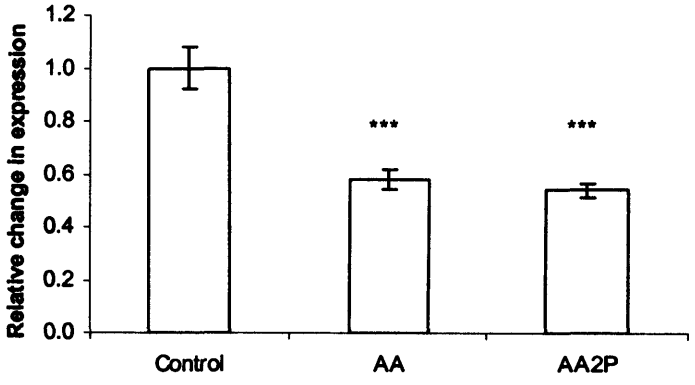


Figure 5.12. Effect of vitamin C on TFRC mRNA expression in GM5659 HDFs. HDFs were incubated with 100 μM AA or AA2P or medium alone for 12 hours. Total RNA was extracted at the end of each treatment and the steady-state levels of TFRC mRNA were measured by real-time RT-PCR. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* control.

The fraction of TFRC expressed at the cell surface is crucial for transferrin binding. Consequently, the effects of vitamin C on the expression of functional TFRC on the cell surface were also investigated. GM5399 HDFs were incubated with 100 μM AA or AA2P, 300 μM DFO, 32.65 μg/ml Fe supplied as FAC or growth medium alone for about 18 hours. The TFRC protein has a proteolytic cleavage site for trypsin. Consequently, treatment of cells with trypsin causes the release of an approximately 75,000 Da polypeptide into the cellular supernatant (Kühn *et al.* 1984). As this was shown to compromise the immunodetection of the receptor at the cell surface (Chen *et al.* 1991), in the present work cells were harvested using an enzyme-free dissociation solution. Expression of the TFRC at the cell-surface was measured by a flow cytometric immunoassay as described in Section 2.13 and in Figure 5.13. As expected (Ward *et al.* 1982; Wiley and Kaplan 1984; Owen and Kühn 1987; Caltagirone *et al.* 2001), HDFs expressed TFRC on the cell-surface and iron chelation with DFO for about 18 hours nearly doubled the expression of the receptor (90.9 ± 9.9 % increase when compared with control, $N = 3$, $P = 0.004$). In contrast, iron overloading with FAC for about 18 hours significantly reduced the levels of TFRC by about one third (32.2 ± 9.8 % decrease when compared with control, $N = 3$, $P = 0.031$). To investigate the effect of vitamin C on the number of cell-

surface receptors, HDFs were incubated with either AA or AA2P (100 μM) for the same period of time. Notably, AA and AA2P significantly reduced cell-surface TFRCs to a similar extent as FAC (35.7 ± 4.5 % decrease when compared with control, N = 3, P = 0.006 and 32.5 ± 3.4 % decrease when compared with control, N = 3, P = 0.003, respectively).

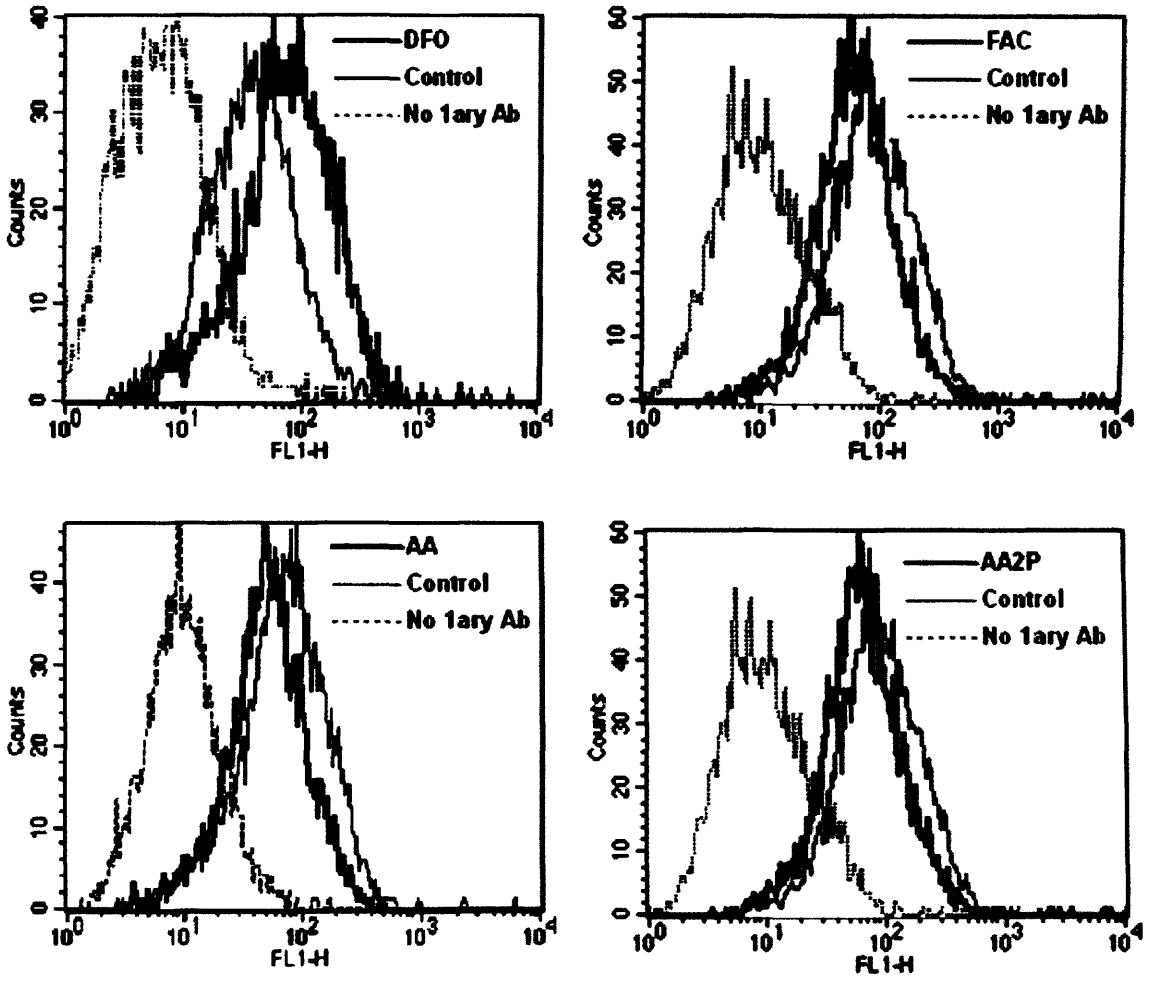


Figure 5.13. Flow cytometry immunoassay of cell-surface TFRC expression. HDFs were incubated with 300 μM DFO, 32.65 μg/ml Fe supplied as FAC, 100 μM AA or AA2P, or growth medium alone (control) for about 18 hours. HDFs were then sequentially incubated with monoclonal anti-human CD71 and FITC-anti-mouse IgG antibodies. The amount of TFRC antigenic sites on the cell surface was expressed as arbitrary FL1 fluorescence units and subsequently converted into a percentage of the fluorescence intensity in control cells within the same experiment. Histograms are representative of 3 independent experiments.

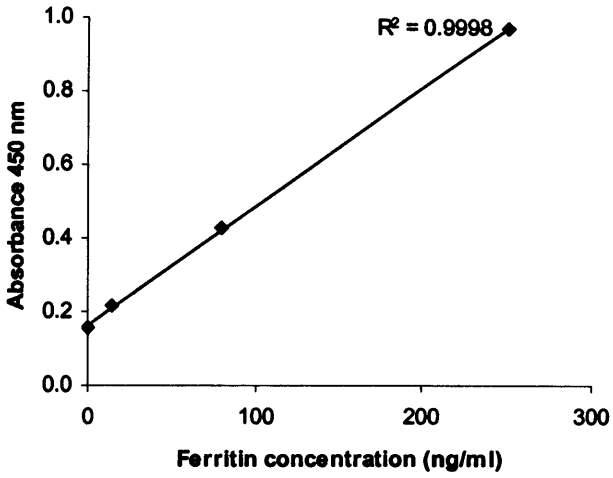
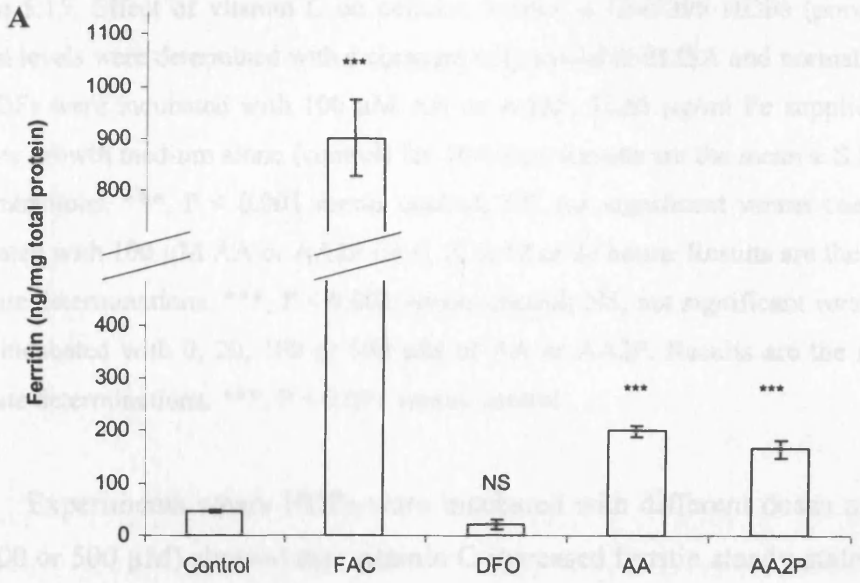


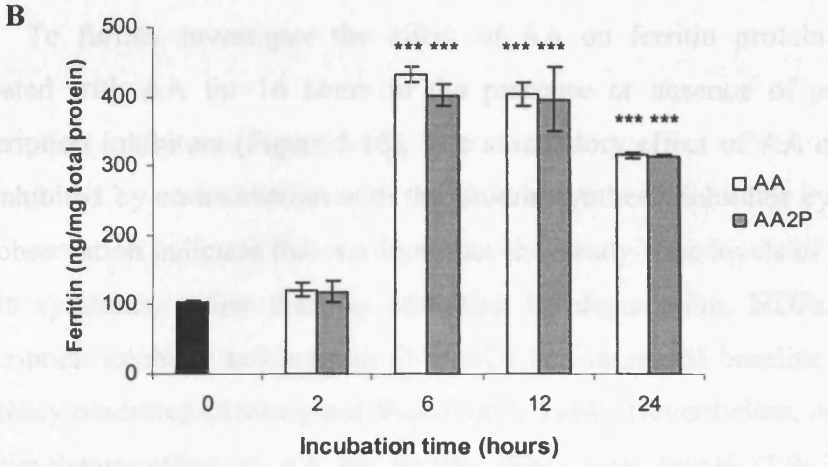
Figure 5.14. Standard curve of ferritin concentration as detected with a commercial ELISA. A standard curve was constructed by linear regression analysis after plotting the mean absorbance of each reference standard against its concentration in ng/ml (0, 15, 80 or 250 ng/ml). According to the supplier, the assay sensitivity is 5.0 ng/ml.

ii) Ferritin expression

Ferritin is the major intracellular iron storage protein and its expression is readily induced in response to iron overloading (Torti and Torti 2002). In the present work, the steady-state levels of cellular ferritin were measured using a commercial ELISA. HDFs were incubated as described below and ferritin concentration in cell lysates was determined from a standard curve and subsequently corrected for total protein. A representative standard curve is shown in Figure 5.14.

As expected, incubation of GM5399 HDFs with 32.65 μg/ml Fe supplied as FAC for 16 hours caused a dramatic (nearly 20-fold) increase in ferritin levels, whereas iron chelation with 300 μM DFO for the same period of time reduced the levels of ferritin by half (Figure 5.15A). To investigate the effect of vitamin C on cellular ferritin, HDFs were incubated with either AA or AA2P (100 μM) for the same period of time. Notably, incubation of HDFs with AA or AA2P increased ferritin steady-state levels by approximately 4-fold ($P < 0.001$). In a separate set of experiments, HDFs were incubated with AA or AA2P (100 μM) for different periods of time (Figure 5.15B). Both vitamin C derivatives caused a time-dependent increase in ferritin steady-state levels, which reached the maximum (approximately 4-fold increase) at 6-12 hours and was still present, though slightly attenuated (3-fold), at 24 hours of incubation.


A
Ferritin (ng/mg total protein)
1100
1000
900
800
400
300
200
100
0
***
NS
***
***
Control
FAC
DFO
AA
AA2P


B
Ferritin (ng/mg total protein)
500
400
300
200
100
0
*** ***
*** ***
*** ***
AA
AA2P
0
2
6
12
24
Incubation time (hours)

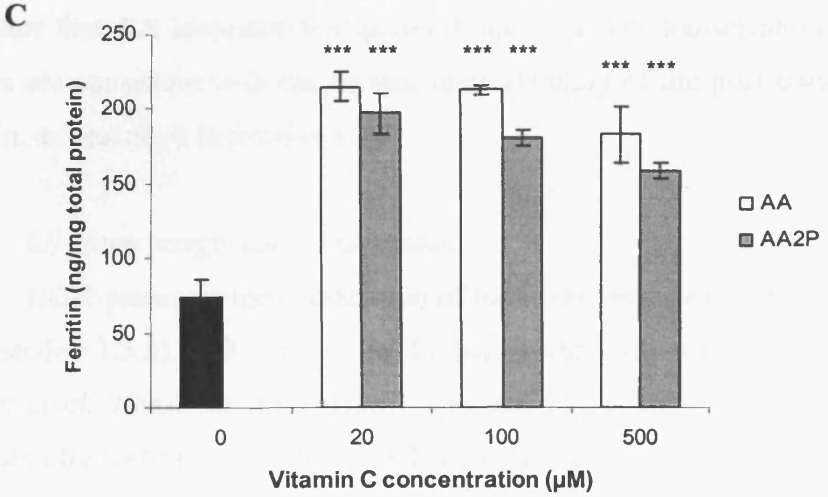

C
Ferritin (ng/mg total protein)
250
200
150
100
50
0
*** ***
*** ***
*** ***
AA
AA2P
0
20
100
500
Vitamin C concentration (μM)

Figure 5.15. Effect of vitamin C on cellular ferritin in GM5399 HDFs (previous page). Cellular ferritin levels were determined with a commercially available ELISA and normalised for total protein. A, HDFs were incubated with 100 μM AA or AA2P, 32.65 μg/ml Fe supplied as FAC, 300 μM DFO or growth medium alone (control) for 16 hours. Results are the mean ± S.D. from 3-6 separate determinations. ***, $P < 0.001$ *versus* control; NS, not significant *versus* control. B, HDFs were incubated with 100 μM AA or AA2P for 0, 2, 6, 12 or 24 hours. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* control; NS, not significant *versus* control. C, HDFs were incubated with 0, 20, 100 or 500 μM of AA or AA2P. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* control.

Experiments where HDFs were incubated with different doses of AA or AA2P (0, 20, 100 or 500 μM) showed that vitamin C increased ferritin steady-state levels at all doses tested, but the effect was not dose-dependent (Figure 5.15C).

To further investigate the effect of AA on ferritin protein levels, HDFs were incubated with AA for 16 hours in the presence or absence of protein synthesis and transcription inhibitors (Figure 5.16). The stimulatory effect of AA on ferritin expression was inhibited by co-incubation with the protein synthesis inhibitor cycloheximide (CHX). This observation indicates that AA increases the steady-state levels of ferritin by increasing protein synthesis, rather than by inhibiting its degradation. HDFs incubated with the transcription inhibitor actinomycin D (ActD) had increased baseline levels of ferritin, as previously described (Rittling and Woodworth 1984). Nevertheless, ActD did not abrogate the stimulatory effect of AA on ferritin steady-state levels (3.9-fold increase), which indicates that AA increases ferritin synthesis by a post-transcriptional mechanism. These results are consistent with the current understanding of the post-transcriptional control of ferritin, as described in Section 1.3.7.

iii) Haem oxygenase-1 expression

HO-1 promotes the degradation of haem and may therefore release iron into the LIP (see Section 1.3.8). HO-1 induction, in turn, occurs primarily by transcriptional activation (Ryter *et al.* 2006). In the present work, the steady-state levels of HO-1 mRNA were measured by real-time quantitative RT-PCR, using HPRT1 as an endogenous control gene. Primers were designed for the specific amplification of the HO-1 mRNA. The amplification

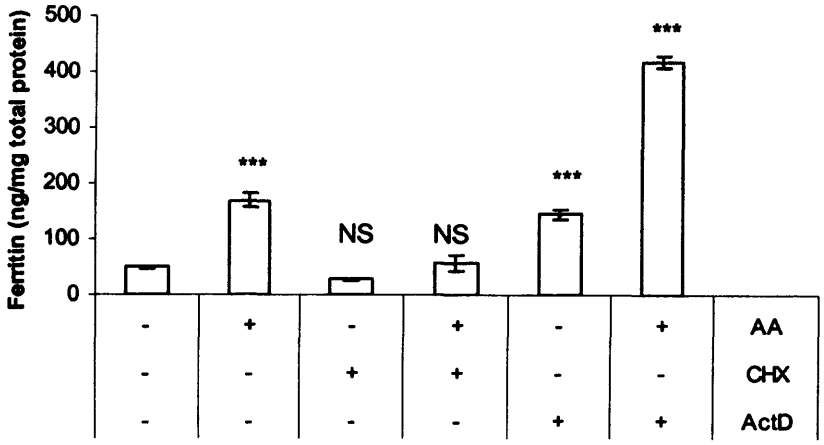


Figure 5.16. Effect of protein synthesis and transcription inhibitors on ferritin induction by AA. HDFs were incubated with 100 μM AA for 16 hours in the presence or absence of 3 μg/ml CHX or 1 μg/ml ActD. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* control; NS, not significant *versus* control.

curves, standard curve and dissociation curves corresponding to the optimised amplification conditions are shown in Figure 5.17. Standard curves generated by linear regression analysis of log standard DNA *versus* Ct were used to calculate reaction efficiency and to estimate target quantity for each individual sample, as described in Sections 2.10.5 and 5.2. The dissociation curves of the amplification products showed a single peak, confirming the specificity of the reaction and the absence of primer dimers. Further confirmation of the reaction specificity was obtained by analysis of the amplification products by gel electrophoresis, which showed a single band with the expected size (Figure 5.18).

To study the effects of vitamin C on HO-1 expression, nearly confluent GM5399 HDFs were incubated with 100 μM vitamin C (AA or AA2P) or growth medium alone for 2, 6, 12 and 24 hours. Both vitamin C derivatives caused a time-dependent decrease in the steady-state levels of HO-1 mRNA (Figure 5.19A). Results were replicated in GM5659 HDFs incubated with AA or AA2P for 12 hours (Figure 5.19B). To investigate the effects of iron loading or chelation on HO-1 mRNA expression, GM5399 HDFs were incubated with FAC or DFO, respectively. The specific iron chelator DFO (300 μM) doubled the levels of HO-1 mRNA ($P < 0.005$) (Figure 5.19C). FAC (32.65 μg/ml Fe) caused a small but significant increase in HO-1 mRNA (1.6 fold; $P < 0.005$) (Figure 5.19C).

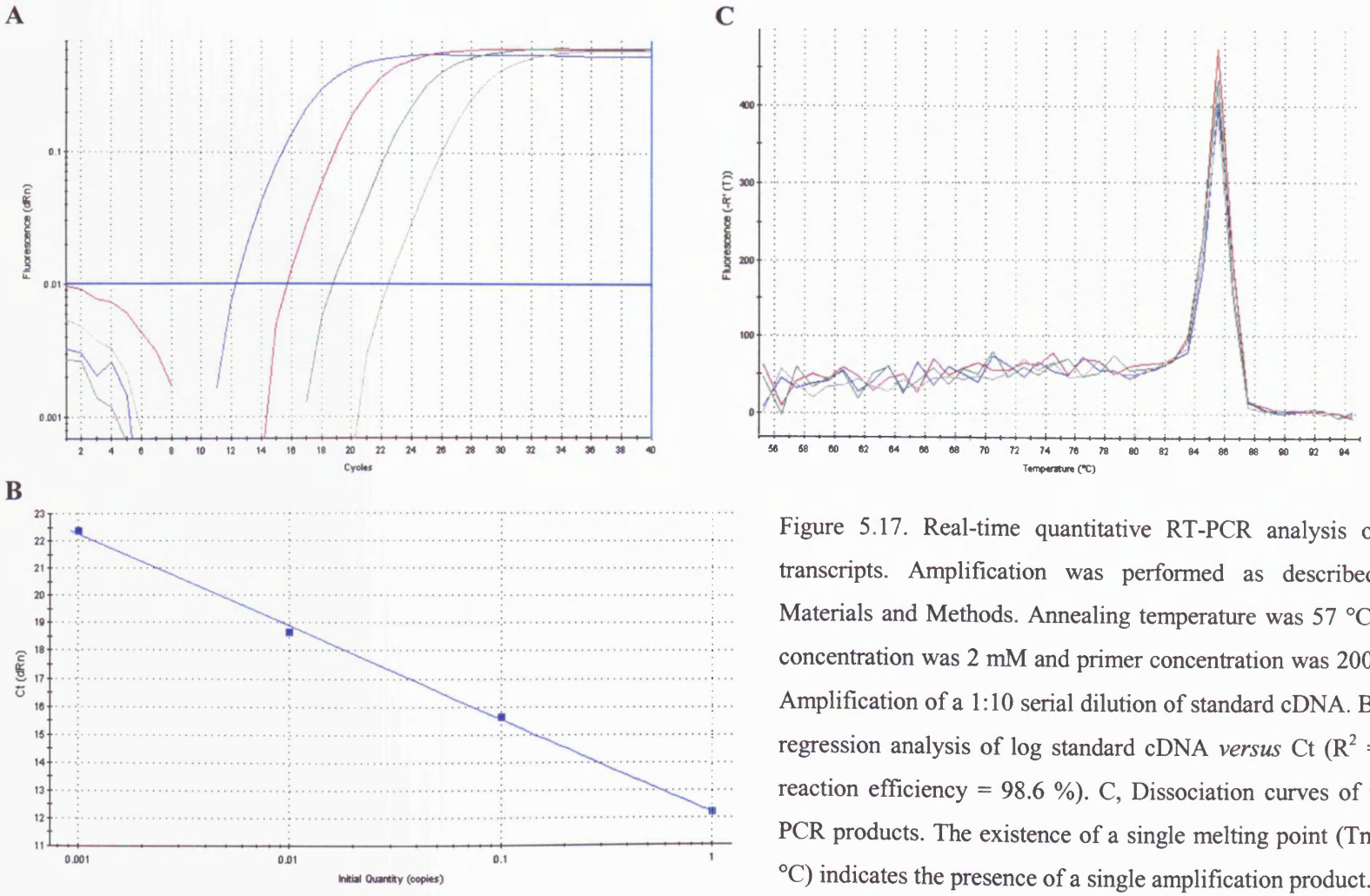


Figure 5.17. Real-time quantitative RT-PCR analysis of HO-1 transcripts. Amplification was performed as described under Materials and Methods. Annealing temperature was 57 °C, $MgCl_2$ concentration was 2 mM and primer concentration was 200 nM. A, Amplification of a 1:10 serial dilution of standard cDNA. B, Linear regression analysis of log standard cDNA *versus* Ct (R^2 = 0.999, reaction efficiency = 98.6 %). C, Dissociation curves of multiple PCR products. The existence of a single melting point (Tm = 85.5 °C) indicates the presence of a single amplification product.

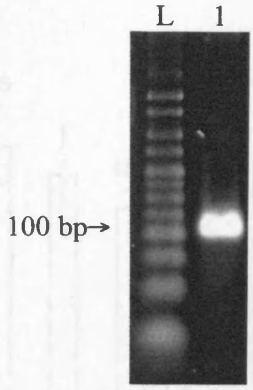
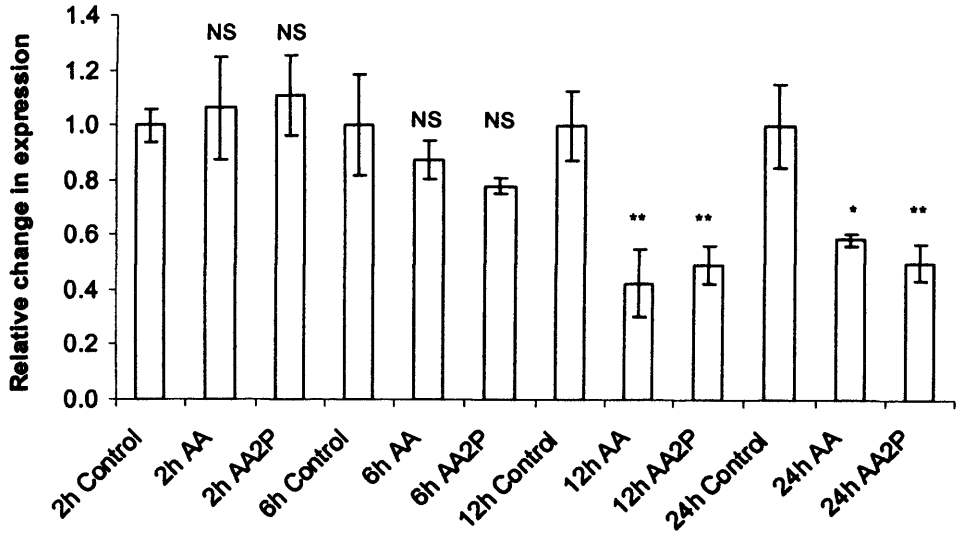


Figure 5.18. Analysis of HO-1 RT-PCR amplification product by gel electrophoresis. An aliquot of PCR product was examined on a 3 % agarose gel. Electrophoresis was run as described in Materials and Methods and the PCR products visualised under UV after EtBr staining. L, DNA HyperLadder V; 1, HO-1.

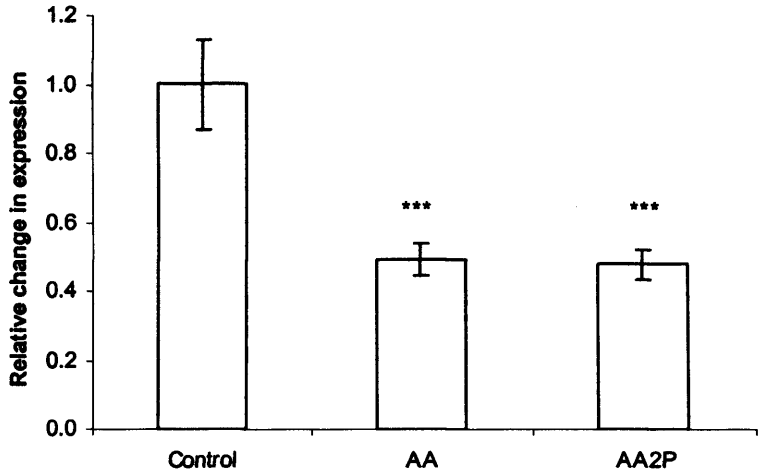
UVA radiation is a well-known inducer of HO-1 expression in HDFs, so the effect of vitamin C on UVA-induced induction of HO-1 expression was also investigated. As expected, HO-1 mRNA expression was dramatically induced by exposure to 15 J/cm^2 UVA radiation (fold change 10.4 ± 2.5) (Figure 5.20). Notably, UVA-induced expression of HO-1 was significantly reduced in HDFs that had been pre-incubated with AA or AA2P ($P < 0.005$).

Figure 5.19. Effect of vitamin C on HO-1 mRNA expression in HDFs (next page). Total RNA was extracted at the end of each treatment and the steady-state levels of HO-1 mRNA were measured by real-time RT-PCR. A, GM5399 HDFs were incubated with 100 μM AA or AA2P or medium alone for 2, 6, 12 and 24 hours. Results are the mean ± S.D. from 3 separate determinations. NS, not significant; *, $P < 0.01$; **, $P < 0.005$ *versus* the time-matched control. B, GM5659 HDFs were incubated with 100 μM AA or AA2P or medium alone for 12 hours. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* control. C, GM5399 HDFs were incubated with either 300 μM DFO, 32.65 μg/ml Fe supplied as FAC or medium alone for 12 hours. Results are the mean ± S.D. from 3 separate determinations. **, $P < 0.005$ *versus* control.

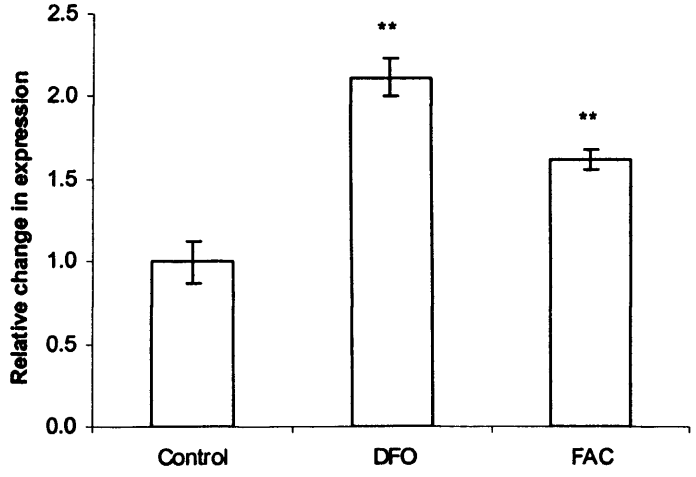
A


Relative change in expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NS
NS
NS
NS
**
**
*
**
2h Control
2h AA
2h AA2P
6h Control
6h AA
6h AA2P
12h Control
12h AA
12h AA2P
24h Control
24h AA
24h AA2P

B


Relative change in expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
Control
AA
AA2P

C


Relative change in expression
2.5
2.0
1.5
1.0
0.5
0.0
**
**
Control
DFO
FAC

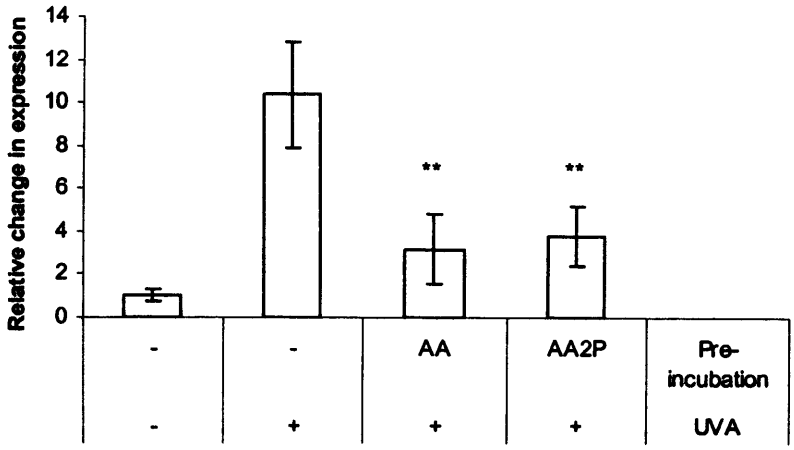


Figure 5.20. Effect of vitamin C on UVA-induced induction of HO-1 expression. HDFs were pre-incubated with 100 μM AA or AA2P or medium alone for 12 hours. Cells were then exposed to 15 J/cm^2 UVA as described in Materials and Methods and subsequently incubated in growth medium at 37 °C for 4 hours. The steady-state levels of HO-1 mRNA were measured by real-time RT-PCR. Results are the mean ± S.D. from 3-5 separate determinations. **, $P < 0.005$ *versus* control.

5.5. Discussion

5.5.1. Effect of vitamin C on intracellular 'chelatable' iron

The majority of the intracellular iron is safely bound to storage, functional or transport proteins, but a small pool of cellular iron is loosely bound to low molecular weight organic chelators. This labile iron pool (LIP) is thought to be available for participating in the formation of highly reactive oxidant species (see Section 1.3.6). The experiments described in Chapter IV showed that vitamin C enhanced iron-dependent, H_2O_2-induced DNA damage and cell death. It could thus be speculated that vitamin C would promote Fenton-type oxidative damage by increasing the availability of intracellular catalytic iron. Relative changes in the intracellular 'chelatable' iron (or LIP) were measured here using an adaptation of the method of Petrat *et al.* (1999). This method is based on quenching the fluorescence of the transition metal indicator PG SK by intracellular iron and its subsequent dequenching upon addition of a strong membrane-permeable iron chelator, BIP. The level of intracellular labile iron is directly proportional to the increase in BIP-induced PG SK fluorescence (Petrat *et al.* 1999).

PG SK is capable of removing iron that is complexed with organic cellular iron chelators (citrate, phosphate, ATP) (Petrat *et al.* 2002). Even though other metals quench the fluorescence of PG SK in solution (Cu^{1+}, Cu^{2+}, Co^{2+} and Ni^{2+}), the intracellular concentration of these metals is very low, and therefore their contribution to fluorescence quenching of PG SK in a cellular context is thought to be negligible (Petrat *et al.* 1999). In addition, the present experiments showed that the increase in fluorescence upon the addition of BIP was markedly reduced by incubation with DFO and increased by FAC, confirming that iron is a major contributor to fluorescence quenching.

The addition of the membrane-permeable transition metal chelator BIP increased the intracellular fluorescence of PG SK in untreated cells by approximately three-fold, showing that the fluorescence of the metal indicator was already partially quenched by iron chelation under control conditions. It can thus be assumed that HDFs have a pool of PG SK-chelatable iron at baseline. Breuer *et al.* (1995) have determined that the intracellular LIP of human erythroleukemia K562 cells is composed mainly of ferrous iron. It is likely that, under normal conditions, iron derived from the endosomes is maintained in the cytosol as a transit pool of chelatable ferrous iron, which is the form of iron that is incorporated in iron-containing proteins (*e.g.* haem, ferritin, ribonucleotide reductase). However, this is a form of iron that can cause cellular damage by participating in Fenton reactions (see Section 1.3.3). PG SK seems to be evenly distributed over the cytosol and organelles and it is an indicator of ferrous iron, since the chelating group of the indicator (1,10-phenanthroline) has higher affinity for bivalent iron ions and BIP is a ferrous iron chelator (Petrat *et al.* 1999).

In a recent study, Sturm *et al.* (2005) reported that co-treatment with AA (150 μM for 4 hours) enhanced the increase in the LIP (calcein-chelatable iron) in human hepatoma HepG2 cells incubated with iron sucrose. The combined incubation with iron sucrose and AA also increased the synthesis of ferritin when compared with iron sucrose alone. The above-mentioned results showed that AA increased the bioavailability of iron from iron sucrose, but the authors did not report effects of AA alone. In the present work, physiological concentrations (100 μM) of AA or AA2P caused significant increases in intracellular PG SK-chelatable iron. The increase in the LIP observed herein occurred in the absence of added external iron, which suggests that AA causes the release of safely-bound

iron into the LIP, ultimately leading to an increase in intracellular chelatable Fe^{2+}. AA could do this by releasing ferrous iron from ferritin. In fact, certain reducing agents can readily mobilise iron from ferritin *in vitro* in the presence of a Fe^{2+} chelating agent (*e.g.* BIP, FZN) (Funk *et al.* 1985, Boyer and McCleary 1987). AA, which is found in many cell types at high concentrations, is both an iron reducer and a chelator (Khan and Martell 1967). AA could diffuse into ferritin, reduce ferric to ferrous iron and chelate it, assisting in its diffusion through the ferritin channels (Tufano *et al.* 1981; Boyer and McCleary 1987). Once outside the ferritin molecule, AA would then be exchanged by a stronger chelator such as BIP (Tufano *et al.* 1981; Boyer and McCleary 1987). To date, the release of iron from ferritin has only been demonstrated *in vitro*. Nevertheless, the fact that vitamin C supplements increase urinary iron excretion during DFO chelation therapy (O'Brien 1974) suggests that the vitamin enhances iron mobilisation *in vivo*.

5.5.2. Effects of vitamin C on iron-regulated gene expression

Mammalian cells control intracellular iron levels tightly by regulating the levels of proteins involved in iron uptake and storage. Ferritin is the major intracellular iron storage protein, limiting the availability of catalytic iron for participation in the formation of oxygen radical species (see Section 1.3.5). On the other hand, TFRCs expressed at the cell surface are crucial for transferrin binding and hence for iron uptake (see Section 1.3.2). The regulation of TFRC and ferritin expression by iron is a well-established post-transcriptional mechanism that is under the control of the IRE/IRP system (see Section 1.3.7). When iron is in excess, ferritin synthesis is induced and the TFRC mRNA is degraded, leading to a decrease in the number of receptors in the cell-surface. The present work compared the effects of AA on TFRC and ferritin expression with those of two well-established modulators of the intracellular LIP and of iron-regulated gene expression, DFO and FAC (Richardson and Baker 1992). The regulation of TFRC and ferritin by iron in HDFs has been previously studied. HDFs of different lineages, including skin fibroblasts, express TFRCs at the cell-surface (Ward *et al.* 1982; Ekblom *et al.* 1983; Wiley and Kaplan 1984; Chen *et al.* 1991), and the number of TFRCs is modulated in response to the changes in cellular iron content (Ward *et al.* 1982). Treatment of HDFs with FAC or hemin increases the intracellular iron content, augmenting ferritin biosynthesis and decreasing the number

of TFRCs (Ward *et al.* 1982, 1984; Knisely *et al.* 1989). Conversely, incubation with DFO chelates intracellular iron pools, leading to a concomitant increase in TFRC biosynthesis and decrease in ferritin biosynthesis (Knisely *et al.* 1989).

The regulation of ferritin expression by iron occurs mainly by a post-transcriptional mechanism, so ferritin protein levels, and not the mRNA levels, were measured in the present study. In fact, increased ferritin H and L mRNAs have been reported in a situation of iron depletion, as indicated by increases in transferrin receptor expression and IRP activation, as well as a decline in ferritin protein (Jiao *et al.* 2006). As expected, HDFs responded to iron overload with a dramatic increase in ferritin levels and a reduction in TFRC mRNA and number of receptors in the cell-surface. Conversely, iron chelation reduced the levels of ferritin by half and doubled the steady-state levels of TFRC mRNA and cell-surface receptor. Notably, incubation of HDFs with a physiological concentration of vitamin C (100 μM AA or AA2P) increased cellular ferritin and down-regulated the TFRC in a time-dependent manner. This is in agreement with the ability of vitamin C to expand the intracellular LIP of HDFs, as described in the previous Section. Furthermore, the results of this work indicate that AA increases ferritin synthesis by a post-transcriptional mechanism, in agreement with the current understanding of the iron-dependent regulation of ferritin by the IRE/IRP system. Indeed, CHX, but not ActD, inhibits the induction of ferritin synthesis by iron *in vivo* (Millar *et al.* 1970).

In this respect, it is worth noting that HDFs incubated with the transcription inhibitor ActD had increased baseline levels of ferritin. The effect of ActD is not understood but is has been previously observed (Rittling and Woodworth 1984). It is probably related with a distinct, iron-independent regulation mechanism of ferritin induction. In fact, ferritin is a well-known acute-phase protein (Konijn and Hershko 1977). The acute-phase response represents a series of physiological changes that are rapidly induced in mammals in response to various stimuli, such as infection or tissue injury, with the purpose of preventing further damage and activating repair processes. The initiation and resolution of the acute-phase response is coordinated by a network of cytokine activation (Baumann and Gauldie 1994). Ferritin synthesis is increased during the acute-phase response (Konijn and Hershko 1977). This activation may be mediated by cytokines

(Feelders *et al.* 1998), is independent from intracellular iron, occurs at the translational level and is not accompanied by any effect on TFRC expression (Rogers *et al.* 1990).

In the studies of Toth *et al.* (1995), AA (150 μM) *per se* did not affect cellular ferritin content in human hepatoma and leukaemia cell lines, but it enhanced ferritin synthesis in response to iron overloading, when cells were treated with FAC. In a subsequent work, the authors reported that AA increased cytosolic aconitase activity (Toth and Bridges 1995). However, having failed to observe a change in ferritin synthesis, the authors assumed that AA activated latent IRP-1 in the cytosol into an aconitase (*i.e.* AA would increase aconitase activity of IRP-1 without releasing it from the IRE in the 5'-UTR of ferritin mRNA). More recently, a high concentration of AA (1 mM) yielded a greater than 2-fold increase in ferritin in lens epithelial cells from dogs' eye without addition of exogenous iron (Goralska *et al.* 1997). As in the present work, AA increased ferritin synthesis mainly by post-transcriptional mechanisms. In fact, the authors observed a 4- to 14-fold increase in *de novo* ferritin synthesis when cells were incubated with AA alone, whereas H- and L-ferritin mRNAs were elevated by just 30 %. Nevertheless, the present study demonstrates the significance of the effect of low, physiological concentrations of vitamin C on cellular ferritin in human cells for the first time.

The fact that AA increases ferritin synthesis by post-transcriptional mechanisms implies that AA has to release IRP from the IRE at the 5'-UTR of the ferritin mRNA. IRP release may occur either directly by oxidation/reduction mechanisms or indirectly through an increase in cellular LIP. AA, however, is an effective reducing agent and reducing conditions are known to increase IRP binding to IRE, so the former is unlikely. The second possible explanation (*i.e.* AA causing the release of IRP from IRE by increasing intracellular LIP) is also compatible with the results discussed in Section 5.4.1.

Under the current experimental conditions, the ability of vitamin C to increase cellular ferritin levels was not dose-responsive. This is in agreement with the results of Goralska *et al.* (1997), who showed that AA increased *de novo* ferritin synthesis in lens epithelial cells from dogs' eye to a similar extent at 150 μM and at 1 mM. In fact, if at all, there was in the present work a small decrease in ferritin levels with increasing doses of AA or AA2P. This suggests that vitamin C modulates IRP binding mainly by increasing levels of intracellular labile iron; however, at high intracellular AA concentrations, the

reducing properties of AA would promote IRP binding to some extent, so that the release of IRP from IRE (and concomitant ferritin expression) may be somehow attenuated. If this is the case, then it would represent an additional mechanism by which high intracellular vitamin C concentrations would be more harmful than normal, 'physiological' levels, as they would limit ferritin synthesis and hence increase the potential for DNA damage to occur. This possibility could be assessed in the future by studying the effects of increasing concentrations of intracellular AA on the binding of IRPs to IREs.

Whilst the present study indicated that AA increased ferritin synthesis by a post-transcriptional mechanism, it is worth noting that the increase in protein synthesis may not be the only way by which AA increases cellular ferritin levels. In K562 erythroleukemia cells, AA increases cellular ferritin levels by retarding the lysosomal degradation of the protein shell, rather than increasing ferritin biosynthesis (Bridges and Hoffman 1986; Bridges 1987).

The effects of AA on TFRC expression in cultured cells have not been previously investigated, with the exception of two inconclusive observations in tumour cell lines. Bridges and Hoffman (1986) incubated K562 erythroleukemia cells with DFO in the presence or absence of 300 μM AA. In the absence of AA, 24-hour incubation with DFO caused a dose-dependent increase in cell-surface TFRCs. However, the effect was less evident in the presence of AA. It was suggested that this would be a result of AA increasing the intracellular LIP. A recent report claimed to observe a reduction of TFRCs on incubation of murine melanoma cells with sodium ascorbate (Kang *et al.* 2005), but the dose used was not physiologically relevant (5-10 mM) and killed the vast majority of cells (> 90 % PI-positive) within 4 hours of incubation. Also, the reduction of TFRC expression was not explained by an increase in intracellular iron levels and was not associated with a decreased stability of TFRC mRNA. Consequently, the reduction in TFRC expression was more likely a consequence of cell death.

The two studies mentioned above refer to tumour cell lines and the results are not conclusive. The effect of physiological concentrations of AA on the expression of the TFRC in normal human cells had not been studied. In the present work, the effects of AA on the steady-state levels of the TFRC mRNA of HDFs were measured by real-time quantitative PCR and the number of receptors in the cell-surface was quantified by a flow

cytometric immunoassay. The present work reports for the first time that a physiological concentration of AA (100 μM) reduces the expression of the functional TFRC at the cell-surface, which was associated with a decrease in the steady-state levels of the respective mRNA. TFRC mRNA half-life is decreased in response to increases in the LIP (Klausner *et al.* 1993), so this observation agrees with the current understanding of the regulation of TFRC by iron through the IRE/IRP system and is an additional indication that AA increases the intracellular LIP.

Taken together, the changes in the expression of ferritin and TFRC demonstrate that AA affects iron homeostasis in human cells, namely by increasing iron bioavailability. This is supported by the observation that guinea pigs fed a vitamin C-free diet have low serum iron levels, as well as reduced ferritin protein levels and increased hepatic TFRC mRNA in the liver (Gosiewska *et al.* 1996). The hypothesis is also supported by the scarce evidence available from human trials. Khumalo *et al.* (1998) have studied the association between leukocyte AA and serum TFRC and ferritin concentrations in 178 human volunteers who were part of an investigation of rural African families with iron overload and of individuals with a history of traditional, high-iron content beer consumption. Subjects were given 1-2 g of vitamin C orally for two consecutive days and blood samples were collected 24 hours after each supplement, as well as 24 hours before the first supplement. Even though the authors did not include a control group receiving placebo, they showed that the increase in leukocyte AA following each vitamin C supplement was associated with a small (~12 % decrease) but highly significant decrease in serum TFRC concentration. In addition, the authors reported a significant increase in serum ferritin, but only in a small subset of individuals with iron deficiency. In a recent study, 36 chronic haemodialysis patients were randomly given either 1 g of sodium ascorbate or placebo intravenously in each of two consecutive haemodialysis sessions. Blood samples were collected before the first dose (baseline), before the second dose (day 3) and four days later (day 7). Intravenous vitamin C supplementation caused a small but highly significant decrease (approximately 15 % and 25 % decrease at days 3 and 7, respectively) in serum TFRC, whereas no changes were observed in the placebo group (Tarng *et al.* 2004).

5.5.3. Effects of vitamin C on haem oxygenase-1 expression

HO-1 is the major stress-inducible protein of human skin fibroblasts exposed to oxidative stress arising from H_2O_2, UVA radiation and heavy metal salts (Keyse and Tyrrell 1989). The process involves transcriptional activation of the gene and leads to an increase in steady-state levels of HO-1 mRNA, which peaks at approximately 4 hours post-exposure (Keyse *et al.* 1990; Guyton *et al.* 1996; Panchenko *et al.* 2000; Offord *et al.* 2002). The induction of HO-1 is thought to be part of an inducible protective response against oxidative damage (Vile *et al.* 1994; Rothfuss *et al.* 2001). Previous studies with HDFs showed that externally added iron (FAC, FAS, *holo*-transferrin) causes a transient increase in HO-1 mRNA levels in HDFs (Panchenko *et al.* 2000). So AA, by increasing intracellular chelatable iron, could subsequently up-regulate the expression of HO-1. This could be relevant because higher levels of HO-1, as well as higher fold induction of HO-1, are associated with improved cellular resistance to oxidative stress (Guyton *et al.* 1996). However, the effects of AA on HO-1 expression had not been reported to date. The present study showed that AA causes a time-dependent decrease in the steady-state levels of HO-1 mRNA. HO-1 is mainly regulated by transcriptional activation, so it can be speculated that the presence of AA in cells may limit its activation during oxidative stress. This possibility was investigated here by exposing cells to a well-known inducer of HO-1 expression, UVA radiation.

UVA radiation penetrates human skin, reaching the dermal layer, and is implicated in skin aging and photocarcinogenesis (Trautinger 2001). Therefore, dermal skin fibroblasts are a valid model to study the potential protective effects of antioxidants against UVA-induced damage. Previous work showed that exposure of dermal HDFs to physiological levels of UVA radiation up-regulates the expression of several stress-response genes, including HO-1, interstitial collagenase (matrix metalloproteinase 1), ICAM-1 and CL100 phosphatase. The production of singlet oxygen by UVA seems to be a crucial event in the activation of these genes (Tyrrell 1999). In agreement with previous reports (Vile and Tyrrell 1993; Tyrrell 1999; Offord *et al.* 2002), HO-1 mRNA levels were dramatically increased following irradiation of dermal HDFs with UVA. The dose used in the present work (15 J/cm^2) corresponds to less than one minimal erythemal dose in human skin and can be easily achieved during exposure to a typical tanning lamp, so it represents a

physiological level of oxidative stress (Vile and Tyrrell 1993; Offord *et al.* 2002). According to a previous study, pre-incubation of HDFs with AA had no effect on UVA-mediated induction of HO-1 mRNA (Offord *et al.* 2002). However, cells were incubated with 14 μM AA for 24 hours before irradiation. The present study clearly showed that AA or AA2P at 100 μM significantly limited UVA-mediated induction of HO-1 expression, which further supports the view that AA limits baseline levels of HO-1. In contrast to TFRC or ferritin, HO-1 expression is not regulated by the IRP/IRE system. In fact, this study showed that, in contrast to AA, both iron supplementation (FAC) and chelation (DFO) significantly increased HO-1 expression.

In human cells, HO-1 expression levels are decreased by agents that induce Bach1 (Kitamuro *et al.* 2003). As described in Section 1.3.8, Bach1 competes with the transcriptional activator Nrf2 and represses HO-1 transcription. The repressor activity of Bach1 is thought to explain the low levels of HO-1 activity at basal conditions (Ogawa *et al.* 2001; Ogawa *et al.* 2002; Sun *et al.* 2002). Bach1 function is regulated by the cell redox state. In this respect, oxidation of conserved cysteine residues in the DNA binding domain causes Bach1 to dissociate from the antioxidant response element in the HO-1 promoter, a mechanism by which ROS could release cells from Bach1-mediated transcriptional repression (Ishikawa *et al.* 2005). It is possible that AA keeps the Bach1 cysteine residues in the reduced state, thus activating the HO-1 transcriptional repressor. However, this possibility has not been investigated to date. The fact that AA represses HO-1 expression in baseline conditions may be advantageous as HO-1 promotes the degradation of cellular haem into free iron, which is potentially toxic to cells. However, HO-1 activity is thought to be part of an inducible protective response against oxidative damage (Vile *et al.* 1994; Rothfuss *et al.* 2001), so the AA-mediated repression of HO-1 induction during oxidative stress could be deleterious.

5.6. Summary

Overall, experiments have shown that AA increased the levels of intracellular chelatable iron. This effect was accompanied by a reduction of the levels of the TFRC and an increase in the synthesis of ferritin, which are thought to occur as part of a regulatory

feedback mechanism. These data clearly demonstrate that AA modulates iron homeostasis in normal human cells.

In addition, AA reduced the basal levels of HO-1 mRNA in a time-dependent manner and significantly limited the induction of HO-1 by UVA. It is not understood, however, whether this effect is related to the ability of AA to modulate iron homeostasis.

CHAPTER VI

ANALYSIS OF VITAMIN C-INDUCED GENE EXPRESSION CHANGES IN HUMAN FIBROBLASTS

6.1. Introductory notes on DNA microarray technology

With the recent completion and annotation of several genome sequences, attention has gradually shifted towards functional genomics, *i.e.* the analysis of genes and their products. New methods have been developed for the high-throughput analysis of large sets of genetic information. This post-genomics era includes techniques for gene expression profiling at the transcript level (DNA microarray analysis), protein level (proteomics) and metabolic level (metabonomics), all of which are associated with advances in bioinformatics.

DNA microarray analysis provides a high-throughput approach to analyse in parallel large sets of genes. The basic principle of microarray technology consists in hybridising a sample representing the transcriptome to an array containing probes for a large number of genes. The hybridised probes emit a fluorescent signal that is indicative of the expression of the respective genes. There are two main types of microarray technology: cDNA microarrays and oligonucleotide microarrays. Regarding the former, methods involving spotting of multiple cDNAs onto a glass surface were initially created to monitor the expression of the corresponding genes. Typically, a test and a reference sample (labelled with distinct fluorescent dyes) are simultaneously hybridised to the array. The gene expression analysis is then undertaken by comparing the fluorescence signal of the two samples at each probe, giving a relative measurement level for each RNA molecule. However, this approach requires the preparation and handling of a large number of clones, PCR products and cDNAs, and has many problems concerning the replication of the results generated (Schena *et al.* 1995; Clarke *et al.* 2004).

An alternative approach was based on hybridisation of a test sample to small, high-density oligonucleotide array (Lockhart *et al.* 1996). Using only the sequence information generated from genes, cDNAs or ESTs, independent oligonucleotides (usually 25-mer) are selected to serve as sensitive, unique, sequence-specific probes (Lipshutz *et al.* 1999). Thousands of oligonucleotides are synthesized directly on glass slides using a photochemical deprotection method, a combination of photolithography and solid-phase DNA synthesis (Pease *et al.* 1994). This light directed synthesis allows the construction of highly dense oligonucleotide probe arrays (Lipshutz *et al.* 1999). The *in situ* synthesis offers a number of other advantages over the spotting of pre-synthesised oligonucleotides, including increased consistency over the whole surface of the array (Southern *et al.* 1999). High-density probe arrays are commercially available

from Affymetrix, covering the genomes of different organisms including human, mouse, rat, *Drosophila melanogaster*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae* and *Escherichia coli* (see Affymetrix website http://www.affymetrix.com/ for details). It is this type of arrays that is used in the present study.

The first step in target preparation for Affymetrix gene expression analysis consists in isolating either T-RNA or mRNA from the sample to be analysed. The first strand of ds-cDNA is then synthesised from the mRNA by using an oligo dT primer (to select for mRNAs containing poly A tails), which also contains at the 5' end the sequence for the transcription start site (promoter) recognised by the RNA polymerase of phage T7. The second strand of the ds-cDNA molecule is then synthesised using RNase H, which introduces nicks in the mRNA, and DNA polymerase I, which synthesises the second strand using the nicked mRNA as a primer and dislodging the remaining RNA as it progresses to the 3' end. In this reaction, the enzyme DNA ligase connects the several extensions of cDNA that have been formed in place of the initial mRNA strand. The T7 RNA polymerase is then used to transcribe biotin-labelled cRNA *in vitro* from the T7 transcription start site present on the ds-cDNA, by incorporating biotin-labelled ribonucleotides (UTPs and CTPs) into the nascent cRNA. The latter step also leads to linear target amplification since several cRNA copies are produced from the same transcription start site. The biotin-labelled cRNAs are then fragmented and hybridised to the arrays. Target binding is detected by staining the array with a fluorescent dye (streptavidin-phycoerythrin conjugate, SAPE) that specifically binds to the biotin in the cRNAs. The fluorescence signal is then augmented by exposing the arrays to biotinylated anti-streptavidin antibody and by re-staining the arrays with SAPE. The probe arrays are then scanned and the fluorescence signal can be analysed by purpose-built software. For each target RNA there are between 11 and 20 pairs of oligonucleotide probes, each probe pair consisting of a perfect match (PM) of the reverse complement and a mismatch (MM) containing a mutated middle base pair that serves as a measure of unspecific signal. The differences between the intensities in the PM and MM are used by purpose-built computer software to judge the reliability of the data obtained from each probe pair and to calculate a qualitative and quantitative measurement (Butte 2002). This type of microarray platform reports absolute expression levels, making it possible to compare samples between different arrays (unlike cDNA microarrays, which report fluorescence intensity ratios).

6.2. Introductory notes on the use of non-dividing HDF populations as a cell culture model

Fibroblasts do not exhibit a rapid proliferating activity *in vivo*, except when exposed to certain stimuli. Therefore, it is possible that a system where cells enter a non-dividing arrested state but still keep the ability to divide when exposed to several stimuli may be more representative of the *in vivo* situation (Dell'Orco 1974). A number of pharmacological agents (*e.g.* mimosine, lovastatin) are used to keep cells in a non-dividing state. However, the use of such agents is known to have side effects like metabolic perturbations and cytotoxicity. In contrast, cells can be kept in a non-dividing state according to two well-established drug-independent methods: serum deprivation and cell contact inhibition (Davis *et al.* 2001). Contact inhibition is achieved by growing cells to a post-confluent density. HDFs grow well in medium supplemented with 10 % FBS but are subject to density-dependent inhibition of growth when the cell monolayer becomes confluent. When this happens, cells arrest in early G_1 phase and stop proliferating but remain able to resume cell division if they are seeded at a lower density (Carpenter and Cohen 1976; Davis *et al.* 2001).

The removal of serum from culture medium often results in cell cycle exit into a state of quiescence (G_0) and has therefore been widely used to synchronise a variety of eukaryotic cell types (Krek and DeCaprio 1995). Studies have shown that HDFs grown to confluence and incubated in low-serum medium (0.5 % FBS) for at least 48 hours enter a long-term G_0/G_1 arrest, with reduced protein synthesis and glucose consumption, but no loss of cell viability or division potential (Dell'Orco 1974; Tobey *et al.* 1988). These non-proliferating cells are metabolically active and able to re-enter the mitotic cell cycle and proliferate when exposed to an appropriate stimulus (*e.g.* 10 % FBS) (Dell'Orco 1974; Lembach 1976; Danesh *et al.* 1999).

6.3. Specific aims of study

It is becoming evident that the so-called antioxidants have other non-antioxidant roles, which may involve regulation of gene expression. Likewise, it has been hypothesised that AA, by acting as either a pro- or an antioxidant, could interfere with redox-sensitive signalling pathways, eventually inducing gene expression. However, apart from the reasonably well characterised stimulatory action on the differentiation of certain mesenchymal cell types (Section 1.4.9), the effects of physiological doses of AA

on gene expression of normal human cells remain elusive. This study aimed at performing a genome-wide analysis of AA-induced gene expression in HDFs.

Gene profiling was performed by high-density oligonucleotide microarray analysis and further validated by real-time RT-PCR analysis. The effects of AA were initially studied in serum starved HDFs and subsequently confirmed using contact-inhibited cells. The ability of AA to modulate the cell cycle of HDFs was also studied by FACS analysis in order to support the gene expression data.

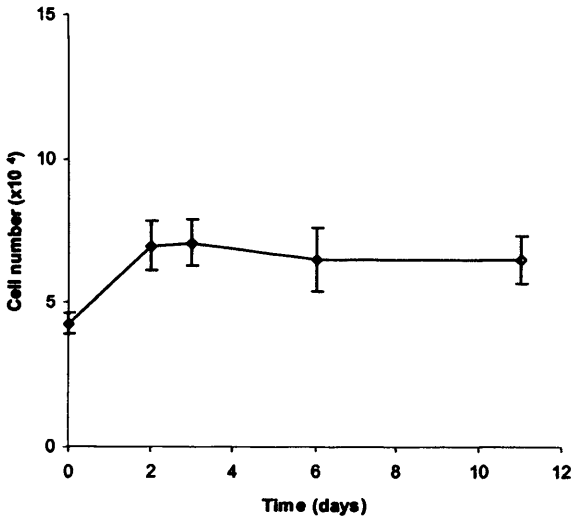
6.4. Results

6.4.1. Effects of serum starvation on cell viability, proliferation and cell cycle distribution

In the present experiments, HDFs were arrested according to the protocols of Dell'Orco (1974) and Tobey *et al.* (1988), with minor changes. Cells were incubated in complete growth medium until approximately 80 % confluent, washed twice with sterile PBS at 37 °C and serum starved by incubation in low-serum medium (0.5 % FBS) for at least 48 hours. HDFs remained viable following 72 hours of incubation in low-serum medium (0.5 % FBS) as judged by their ability to exclude trypan blue (93.8 ± 2.5 % viable cells, N = 5). Experiments with GM5399 cells showed that HDFs could be kept in low-serum medium as a confluent cell monolayer for up to at least 11 days without a decrease in viable cell number (Figure 6.1A). Furthermore, HDFs were able to resume exponential growth when re-plated at lower density and returned to growth medium (Figure 6.1B), as expected (*e.g.* Lembach 1976).

Previous studies have shown that primary skin fibroblasts obtained from healthy subjects are made quiescent by serum starvation for 48 hours and that > 90 % of the cells are arrested at the G_0/G_1 phase of the cell cycle population (Danesh *et al.* 1999). In the present study, FACS analysis was employed to confirm that serum starved fibroblasts were efficiently arrested at the G_0/G_1 phase of the cell cycle. Experiments with GM969 (Figure 6.2A) and GM5659 (Figure 6.2B) showed that, with the serum starvation protocol used in the present study, > 94 % of the cell population was in the G_0/G_1 phase of the cell cycle by 48 hours of serum starvation.

A



B

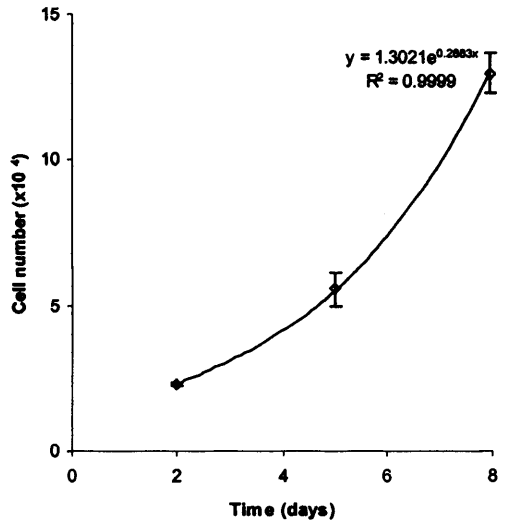


Figure 6.1. Effect of serum starvation on the proliferation of HDFs. GM5399 cells were plated in 6-well plates at a density of 10^4 cells/cm^2. When nearly confluent, cells were incubated in low-serum medium (0.5 % FBS) for up to 11 days. Medium was changed once. A, Cells were trypsinised and counted at 0, 2, 3, 6 and 11 days using the CASY Cell Counter. Results are the mean ± S.D. from 3 separate determinations. B, Following 11 days of incubation in low-serum medium, cells were harvested and re-plated at a ratio of 1:3. Cells were then incubated in growth medium (10 % FBS) and harvested for cell counting at the end of 2, 5 or 8 days. Results are the mean ± S.D. from 3 separate determinations.

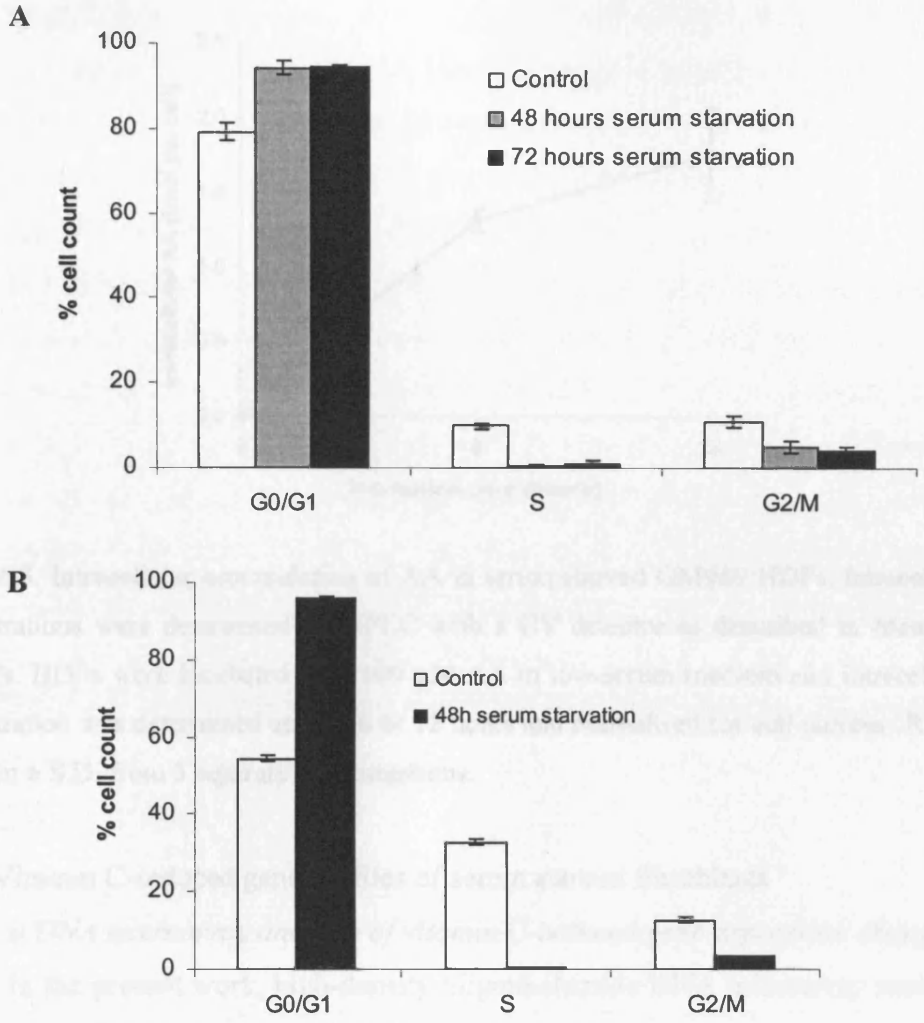


Figure 6.2. Effect of serum starvation on cell cycle distribution of HDFs. Nearly confluent HDFs were incubated with low-serum medium (0.5 % FBS) for 48 or 72 hours. Cell cycle distribution was determined by FACS. A, GM969 HDFs; results are the mean ± S.D. from 5 to 7 separate determinations. B, GM5659 HDFs; results are the mean ± S.D. from 3 separate determinations.

6.4.2. Vitamin C uptake in serum starved fibroblasts

The concentration of intracellular AA in serum starved HDFs was determined by HPLC with UV detection, as described in Materials and Methods. A representative calibration curve was shown in Figure 3.2A (Chapter III). Baseline ascorbic acid levels were below the detection limit of the assay. When incubated with 100 μM AA in low-serum, serum starved HDFs were able to take up AA from the medium and to accumulate it intracellularly in a time-dependent manner (Figure 6.3). A similar effect was described for HDFs incubated in complete growth medium (Section 4.2.1).

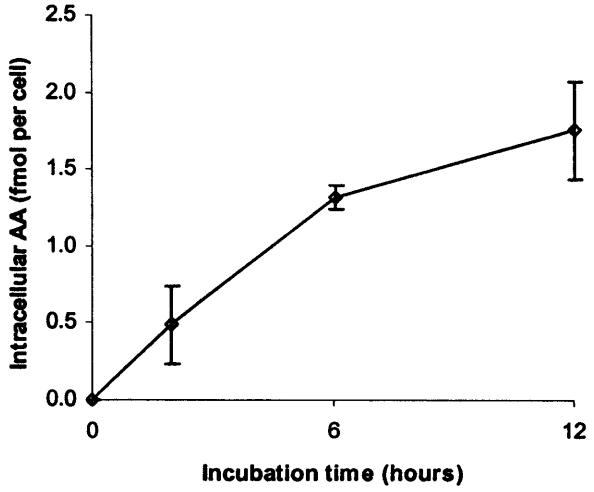


Figure 6.3. Intracellular accumulation of AA in serum starved GM969 HDFs. Intracellular AA concentrations were determined by HPLC with a UV detector as described in Materials and Methods. HDFs were incubated with 100 μM AA in low-serum medium and intracellular AA concentration was determined at 0, 2, 6 or 12 hours and normalised for cell number. Results are the mean ± S.D. from 3 separate determinations.

6.4.3. Vitamin C-induced gene profiles of serum starved fibroblasts

i) DNA microarray analysis of vitamin C-induced gene expression changes

In the present work, high-density oligonucleotide DNA microarray analysis was employed to investigate the expression profiles of GM969 HDFs in response to 100 μM AA, a concentration that can be found in the plasma of humans following vitamin C supplementation (see Section 1.4.3). For the experiments, nearly confluent monolayers were cultured in low-serum medium (0.5 % FBS) for 48 hours to induce a G_0/G_1 arrest and then incubated with AA dissolved in low-serum medium for 8 or 24 hours. The experiments described in Sections 6.4.1 showed how serum starvation was used to keep fibroblasts in a quiescent, non-proliferating status, in which cells remained viable and able to resume cell growth if exposed to a mitogenic stimulus. Results presented in Section 6.4.2 showed that serum-starved HDFs accumulated AA intracellularly in a time-dependent manner. The current experiment was performed twice and, in each independent experiment, serum starved HDFs without AA were used as a control. T-RNA was isolated, rather than mRNA, since this represents a more straightforward method and avoids the loss of sample material. The integrity of the T-RNA extracted from cells was determined by processing samples in the Agilent 2100 Bioanalyzer using

the Eukaryote Total RNA Nano assay (Agilent Technologies, Palo Alto, California, USA). The high-quality of the T-RNA samples was evidenced by the existence of two well-defined peaks in the electropherogram, corresponding to the 18S and 28S ribosomal RNAs, and the absence of low molecular mass degradation products. A representative electropherogram is shown in Figure 6.4.

Biotin-labelled cRNA was subsequently prepared as described in Materials and Methods. Sample quality was determined by denaturing gel electrophoresis. This RNA is complementary to the mRNA present in the cell at that time of extraction, so the presence of several sized RNA species would be expected. This was confirmed by the existence of an RNA smear ranging between 500-5000 bases and centred at approximately 1500 bases (Figure 6.5). An additional step in sample quality control was to determine the efficiency of cRNA fragmentation prior to array hybridisation. According to the Affymetrix Gene Expression manual, thermal and chemical fragmentation generates cRNA fragments of less than 250 bases, which are appropriate for hybridisation to the genome arrays. This was confirmed by denaturing gel electrophoresis (Figure 6.6).

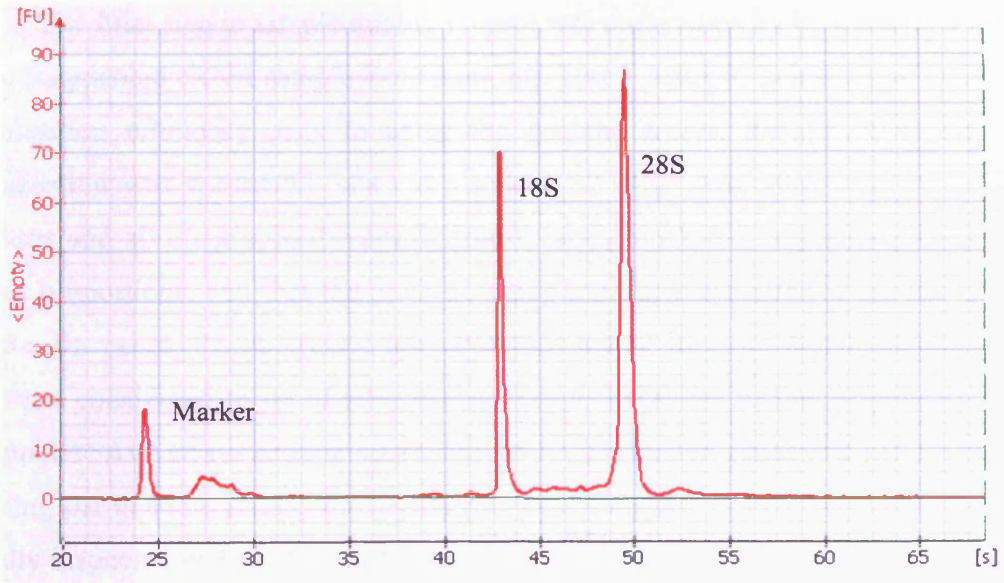


Figure 6.4. Electropherogram of a T-RNA sample obtained using the Agilent 2100 Bioanalyzer. Trace is representative of those obtained for all samples.

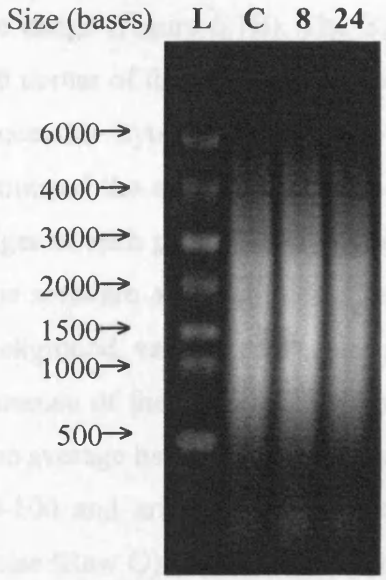


Figure 6.5. Size distribution of biotin-labelled cRNA. RNA ladder (L) and cRNA obtained from control (C) cells or from cells incubated with AA for 8 or 24 hours were analysed by denaturing gel electrophoresis as described in Materials and Methods.

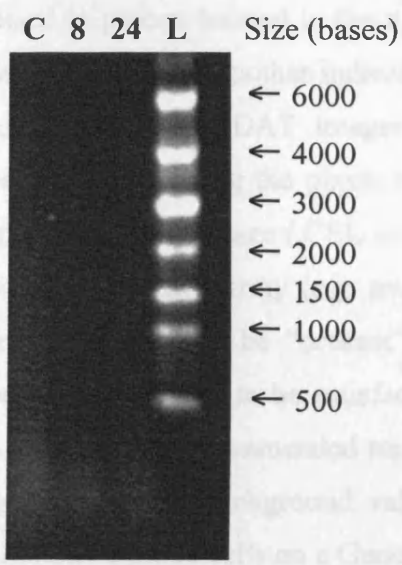


Figure 6.6. Size distribution of fragmented biotin-labelled cRNA. Fragmented cRNA obtained from control (C) cells or from cells incubated with AA for 8 or 24 hours, and RNA ladder (L) were analysed by denaturing gel electrophoresis as described in Materials and Methods.

The final step in sample quality control was undertaken by hybridising a fraction of the fragmented cRNA sample to a GeneChip Test 3 Array to assess target quality and hybridisation efficiency prior to using the genome arrays. Results showed that the hybridisation was successful, since the spiked B2 oligo was present in the expected locations and all other spiked controls (bioB, bioC, bioD and cre) were present at the correct proportions, and that the 3' to 5' ratios were approximately 1 for the GAPDH and β-actin genes. These criteria are explained below in more detail. Samples were, therefore, considered to be of adequate quality to hybridise to the Affymetrix human genome U95Av2 arrays containing probes for 12,500 known genes and 125 ESTs. After scanning the genome arrays, the highly pixelated images (.DAT image) obtained were visually inspected and confirmed free of any image artefacts (*i.e.* high or low intensity spots, scratches, high regional or overall background) (Figure 6.7A). Visual inspection also confirmed that the B2 oligo, which is spiked into each hybridisation cocktail and serves as a positive control for the hybridisation, was present at the boundaries and corners of the array and was correctly used by the software to place an aligned grid over

the image (Figure 6.7B). The B2 oligo also hybridised to probes located in the upper-left corner of the array that define the array name, which represents another indicator of successful hybridisation. The Affymetrix software analysed the .DAT images and estimated the average signal intensity of each gene probe, excluding the pixels in the edges of each probe. The result is a modified, non-pixelated array image (.CEL image). The software also determines quality control information for each array (*e.g.* average background values, noise, the percentage of genes determined to be "present", the presence of the eukaryotic controls and 3'/5' ratios), which proved to be satisfactory. The average background values ranged between 94.2 and 108.7 (recommended range is 20-100 and arrays being compared should ideally have similar background values). Noise (Raw Q) is a measure of the pixel-to-pixel variation of probe cells on a GeneChip array and array data (especially those of replicates) should ideally have comparable Noise values. In the current experiments, the noise values ranged between 3.48-3.97. The percentage of the genes in the array that is determined to be present in the sample, although variable between samples (as a consequence of cellular response to certain stimuli), is expected to be similar between replicate samples. In fact, in the current experiments the percentage of genes determined to be present ranged between 43.2-47.6 in all samples. The eukaryotic hybridisation controls, spiked into each hybridisation cocktail, comprise a mixture of biotin-labeled cRNA transcripts of bioB, bioC, bioD (genes involved in the biotin synthesis pathway of *E. coli*) and cre (the recombinase gene from P1 bacteriophage) prepared in staggered concentrations (1.5 pM, 5 pM, 25 pM, and 100 pM final concentrations for bioB, bioC, bioD, and cre, respectively) and are therefore used to evaluate sample hybridisation efficiency on eukaryotic gene expression arrays. The signal levels for these controls were determined to be present in all the arrays. Finally, the GeneChip expression arrays contain probes for the housekeeping genes GAPDH and β-actin, and the results obtained for these are used to assess the quality of both the RNA sample and of the assay; namely, there are sets of probes for the 3', middle (M') or 5' parts of the sense strand (*i.e.* the one corresponding to the mRNA) for each of these genes. The ratio of signal values of the 3' probe set to the 5' probe should ideally be 1, whereas a high 3' to 5' ratio may indicate degraded RNA or inefficient transcription of ds-cDNA or biotinylated cRNA. In the current experiments, the 3' to 5' ratios of GAPDH and β-actin ranged between 1.01-1.06 and 1.22-1.44, respectively, which is acceptable.

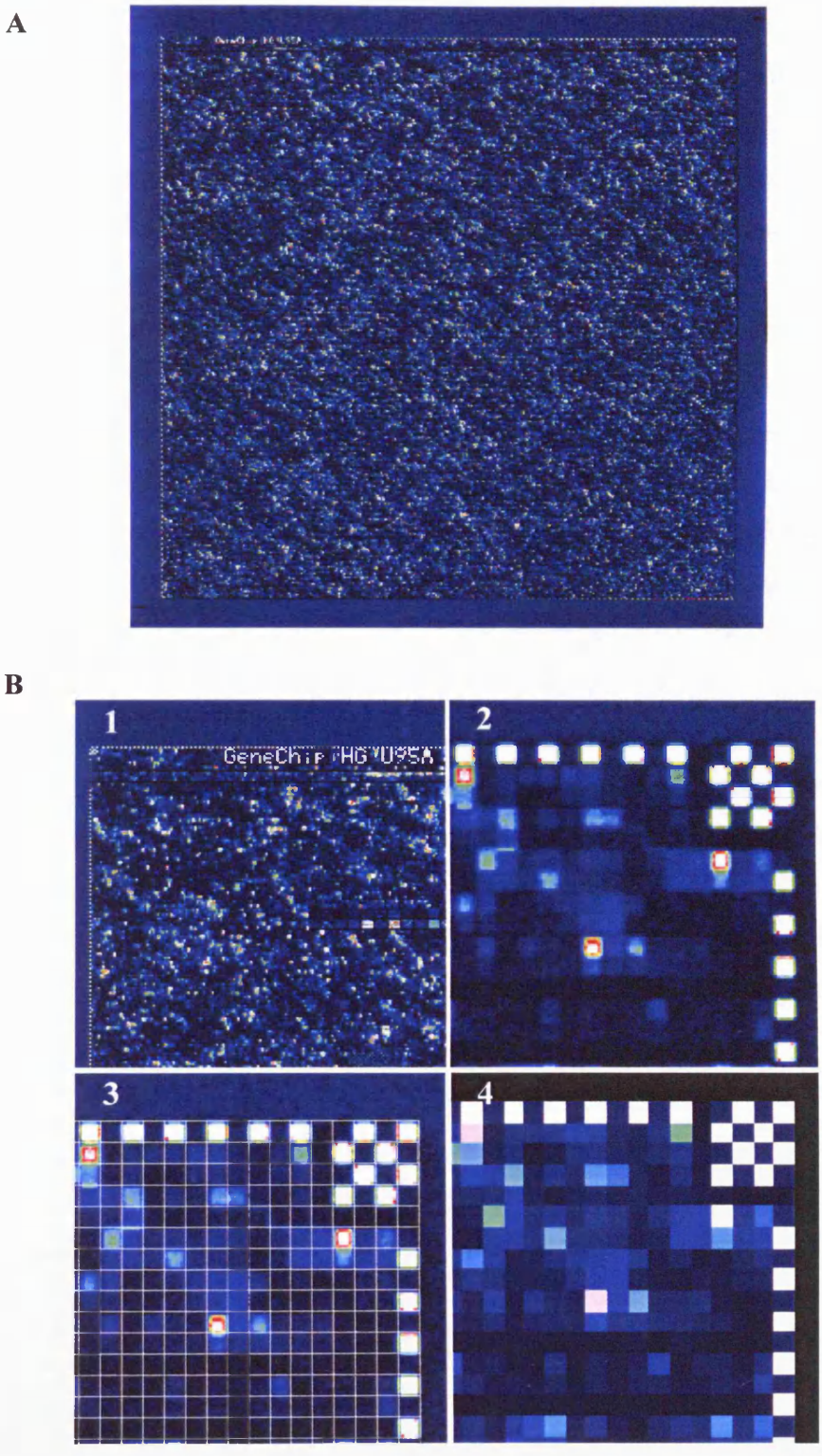


Figure 6.7. Visual inspection of an Affymetrix human genome U95Av2 array. A, full array .DAT image. B, zoomed in .DAT images, highlighting the hybridisation of B2 oligo to probes located in the array boundaries and corners, defining the array name (1) and a checkerboard pattern (2) that the software uses to place an aligned grid over the image (3), as well as the .CEL image obtained after averaging signal intensity in each gene probe.

DNA-Chip Analyser (dChip) software was used to normalise arrays, detect outliers and calculate model-based expression values from the differences between perfect match and mismatch probes in the cell intensity files (Li and Wong 2001). In addition, the software was used to filter a set of genes with altered levels of expression during the time-course of exposure to AA. Genes were determined to have altered expression levels if they had the same change (increase or decrease) in both independent replicate experiments when compared to the respective control and if the averaged fold change of expression was ≥ 1.9 or ≤ -1.9, as described in Materials and Methods (section 2.9.7). A total of 72 genes and ESTs were filtered according to these criteria.

AA increased the expression of 42 genes (Table 6.1). From 8 hours of exposure onwards, AA up-regulated a cluster of 12 genes involved in lipid metabolism. As depicted in Figure 6.8, this cluster includes a gene involved in cholesterol uptake (low-density lipoprotein receptor, LDLR) and genes encoding enzymes involved in all the steps of *de novo* cholesterol biosynthesis pathway: acetyl-coenzyme A acetyltransferase 2 (acetoacetyl Coenzyme A thiolase) (ACAT2), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), isopentenyl-diphosphate delta isomerase (IDI1), farnesyl diphosphate synthase (FDPS), squalene epoxidase (SQLE), sterol-C4-methyl oxidase-like (SC4MOL) and 24-dehydrocholesterol reductase (DHCR24). The cluster also includes a gene involved in fatty acid biosynthesis (fatty acid synthase, FASN) and a gene involved in the regulation of the expression of cholesterol and fatty acids synthesis (insulin-induced gene 1, INSIG1).

At 24 hours of exposure, AA induced a set of 10 genes that are involved in DNA replication or in the control or execution of the progression through the G_2/M phases of the mitotic cell cycle (Table 6.1). Topoisomerase (DNA) II alpha 170kDa (TOP2A) and GINS complex subunit 1 (GINS1) are involved in DNA replication. The genes that regulate progression through G_2/M phases are cyclin B1 (CCNB1), CDC28 protein kinase regulatory subunit 2 (CKS2) and cell division cycle 2 (CDC2). Genes that execute progression through G_2/M phases are those encoding chromosome segregation and spindle associated proteins, such as BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast) (BUB1B), the homolog of microtubule-associated TPX2 from *Xenopus laevis* (TPX2), microtubule-associated protein, RP/EB family, member 2 (MAPRE2), structural maintenance of chromosomes 4 (SMC4) and kinetochore associated 2 (KNTC2), as well as TOP2A.

Table 6.1. Genes with up-regulated expression in response to 100 μM AA (8 or 24 hours treatment in low-serum medium, each in duplicate) in GM969 HDFs.

Probe set ID	*Gene ID*	*Gene Name (official symbol)*	*Fold Change*	
			8 hours	*24 hours*
		Lipid metabolism		
36658_at	1718	24-dehydrocholesterol reductase (DHCR24)	1.8/2.0	2.6/1.4
39328_at	3156	3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR)	2.2/2.7	2.4/1.8
34790_at	39	Acetyl-coenzyme A acetyltransferase 2 (acetoacetyl Coenzyme A thiolase) (ACAT2)	2.8/4.1	4.1/2.7
37325_at	2224	Farnesyl diphosphate synthase (FDPS)	1.4/1.9	2.4/1.5
39799_at	653327	Similar to Fatty acid-binding protein, epidermal (E-FABP) (Psoriasis-associated fatty acid-binding protein homolog) (PA-FABP)	2.0/2.4	1.9/1.6
38429_at	2194	Fatty acid synthase (FASN)	1.7/2.2	2.6/1.1
35303_at	3638	Insulin-induced gene 1 (INSIG1)	3.3/4.3	4.6/1.9
36985_at	3422	Isopentenyl-diphosphate delta isomerise (IDI1)	1.9/2.5	2.2/1.6
38098_at	23175	Lipin 1 (LPIN1)	-1.1/1.4	2.0/2.1
32855_at	3949	Low density lipoprotein receptor (LDLR)	2.1/4.2	3.9/1.8
35839_at	6713	Squalene epoxidase (SQLE)	2.0/3.1	2.5/1.5
33370_r_at	6307	Sterol-C4-methyl oxidase-like (SC4MOL)	2.6/3.3	2.4/1.5
		DNA replication and G_2/M phase		
40145_at	7153	Topoisomerase (DNA) II alpha 170kDa (TOP2A)	-1.1/-1.2	2.9/1.8
39677_at	9837	GINS complex subunit 1 (Psf1 homolog) (GINS1)	-1.1/1.5	2.4/2.0
35699_at	701	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast) (BUB1B)	1.0/1.1	2.5/1.9
40690_at	1164	CDC28 protein kinase regulatory subunit 2 (CKS2)	-1.0/1.5	1.9/1.9
33324_s_at	983	Cell division cycle 2 (CDC2)	-1.3/1.0	1.9/2.0
39109_at	22974	TPX2, microtubule-associated, homolog (*Xenopus laevis*) (TPX2)	-1.0/1.3	2.6/1.5

34736_at	891	Cyclin B1 (CCNB1)	-1.1/1.2	3.2/1.6
37406_at	10982	Microtubule-associated protein, RP/EB family, member 2 (MAPRE2)	1.5/2.1	2.4/3.6
34878_at	10051	Structural maintenance of chromosomes 4 (SMC4)	1.4/1.9	1.7/2.7
40041_at	10403	Kinetochore associated 2 (KNTC2)	-1.4/1.0	3.9/2.0
		Others		
33420_g_at	8539	Apoptosis inhibitor 5 (API5)	1.8/2.2	1.6/-1.2
33331_at	7851	Mal, T-cell differentiation protein-like (MALL)	2.5/2.6	1.1/2.0
39411_at	25976	TCDD-inducible poly(ADP-ribose) polymerase (TIPARP)	2.0/1.9	1.6/1.6
39518_at	133619	Proline-rich coiled-coil 1 (PRRC1)	1.8/2.6	1.5/-1.1
41801_at	26234	F-box and leucine-rich repeat protein 5 (FBXL5)	1.9/3.4	3.6/3.7
1801_at	580	BRCA1 associated RING domain 1 (BARD1)	1.1/1.3	1.8/2.3
173_at	1016	Cadherin 18, type 2 (CDH18)	1.0/1.4	1.7/2.8
2061_at	3676	Integrin, alpha 4 (ITGA4)	1.1/1.3	1.8/3.1
36065_at	9079	LIM domain binding 2 (LDB2)	2.1/2.9	1.4/2.6
743_at	4675	Nucleosome assembly protein 1-like 3 (NAP1L3)	2.5/2.5	2.1/1.3
32143_at	116039	Odd-skipped related 2 (*Drosophila*) (OSR2)	-1.1/1.1	1.9/2.0
36890_at	5493	Periplakin (PPL)	-2.2/-2.4	2.0/3.2
38291_at	5179	Proenkephalin (PENK)	2.0/2.3	1.6/1.0
37185_at	5055	Serpin peptidase inhibitor, clade B (ovalbumin) 2 (SERPINB2)	2.1/2.9	1.0/1.7
36487_at	6474	Short stature homeobox 2 (SHOX2)	1.0/1.5	1.7/2.4
38268_at	6505	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1 (SLC1A1)	1.1/1.3	1.6/2.3
35285_at	8671	Solute carrier family 4, sodium bicarbonate cotransporter, member 4 (SLC4A4)	1.1/1.5	1.7/2.2
41354_at	6781	Stanniocalcin 1 (STC1)	1.5/1.2	2.7/2.0
33982_f_at	7594	Zinc finger protein 43 (ZNF43)	1.1/1.5	1.8/2.1
38717_at	25840	Methyltransferase like 7A (METTL7A)	1.3/1.6	2.2/4.9

Probe set ID according to Affymetrix. Gene ID, name and official symbol according to Entrez Gene.

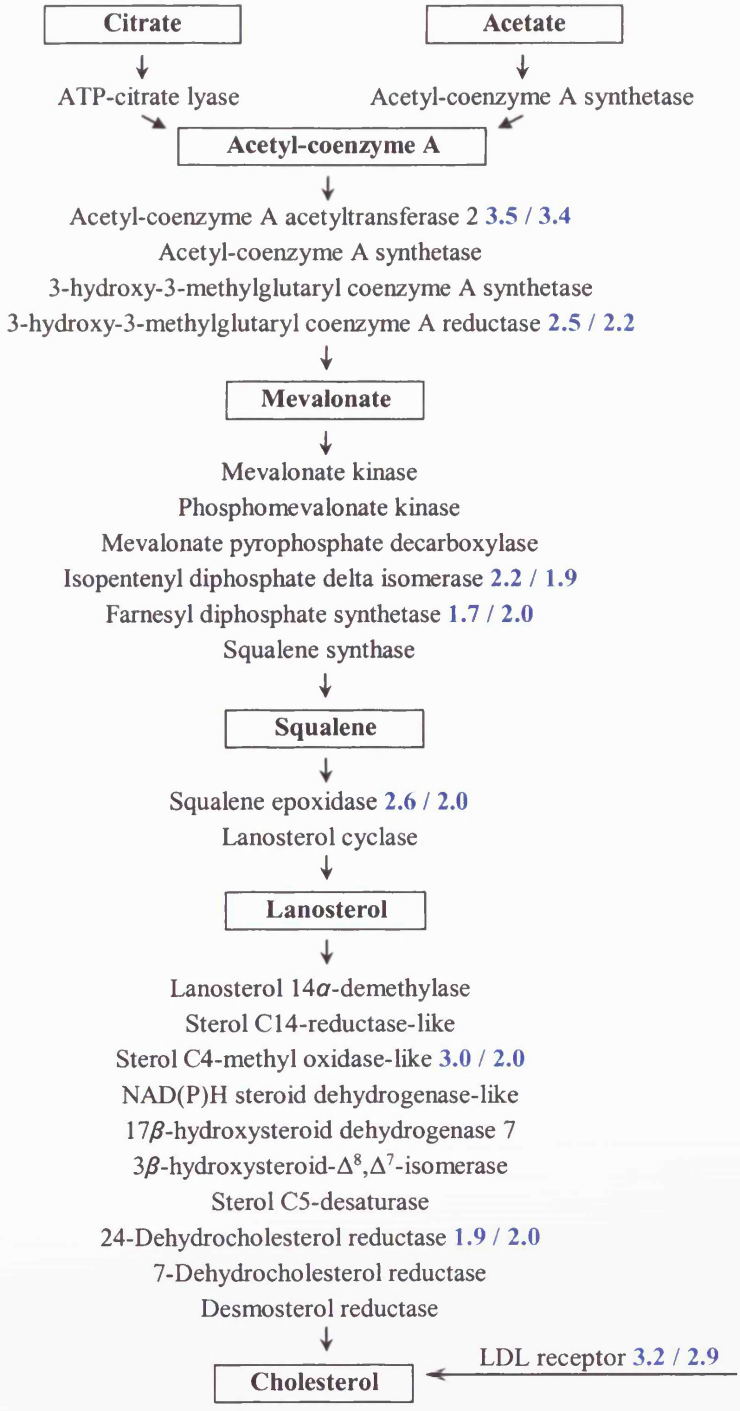


Figure 6.8. Effect of AA on the expression of genes encoding cholesterologenic enzymes in serum starved HDFs as determined by DNA microarray analysis. Cells were incubated with 100 μM AA dissolved in low-serum medium for 8 or 24 hours. Regulation is presented in blue as the averaged fold changes at 8 and 24 hours. The cholesterol biosynthesis pathway diagram was adapted from Horton *et al.* (2003).

Table 6.2. Biological function enrichment in the list of genes up-regulated by AA

Biological function categories	Study categories hits	Study gene list	Total number of genes in category	Total number of genes analysed	Significance
Lipid metabolism[1]	12	42	413	8404	P<0.000001
Cholesterol metabolism[2]	8	42	57	8404	P<0.000001
Mitosis[3]	9	42	121	8404	P<0.000001
Phosphoinositide-mediated signaling[4]	4	42	69	8404	P<0.000001

[1], LDLR, SC4MOL, ACAT2, SQLE, DHCR24, IDI1, FDPS, FASN, HMGCR, PA-FABP, INSIG1, LPIN1. [2], LDLR, SC4MOL, ACAT2, SQLE, DHCR24, IDI1, FDPS, HMGCR. [3], CDC2, CCNB1, CKS2, SMC4, BURB1, MAPRE2, TPX2, KNTC2, TOP2A. [4], BURB1, KNTC2, TOP2A, CKS2.

The genes mentioned above were empirically grouped into functional categories. It is known that groups of co-regulated transcripts, which are under the control of the same *cis*-regulatory elements, tend to take part in common biological processes (Tavazoie *et al.* 1999). It was therefore judged useful to test whether the cluster of up-regulated genes was significantly enriched for genes belonging to different functional categories (*i.e.* if the number of genes in an expression cluster with a given biological function was greater than the number expected by random chance), as described in Materials and Methods (section 2.10.7). The most highly enriched functional categories are represented in Table 6.2. The cluster of genes up-regulated by AA was significantly enriched for genes involved in lipid metabolism. Eight of these genes are part of the cholesterol synthesis pathway. The up-regulation of mitotic genes was also highly significant. Furthermore, there was a significant enrichment in genes that are targets of phosphoinositide-mediated signalling, all of which are also 'mitotic' genes.

AA has also repressed the expression of 30 genes (Table 6.3). A gene involved in iron homeostasis, TFRC, was down-regulated 3-fold. In addition, AA reduced the expression of 4 aminoacyl-tRNA synthetases and genes involved in cellular metabolism. The most down-regulated genes were asparagine synthetase (ASNS), CCAAT/enhancer binding protein gamma (CEBPG), cystathionine-beta-synthase (CBS), methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), mitochondrial phosphoenolpyruvate carboxykinase 2 (PCK2) and dehydrogenase/reductase 3 (DHRS3). The biological processes that were significantly down-regulated by AA were cellular biosynthesis, carboxylic acid metabolism, amino acid metabolism and tRNA aminoacylation (Table 6.4).

Table 6.3. Genes with down-regulated expression in response to 100 μM AA (8 or 24 hours treatment in low-serum medium) in GM969 HDFs.

Probe set ID	*Gene ID*	*Gene Name (official symbol)*	*Fold Change*	
			8 hours	*24 hours*
		Iron metabolism		
37324_at	7037	Transferrin receptor (TFRC)	-3.8/-2.9	-1.4/-1.6
		Metabolism and protein synthesis		
41235_at	468	Activating transcription factor 4 (ATF4)	-3.1/-2.4	-2.4/1.1
39158_at	22809	Activating transcription factor 5 (ATF5)	-3.3/-2.3	-4.3/-1.3
36671_at	440	Asparagine synthetase (ASNS)	-2.5/-2.0	-10.5/-2.5
39219_at	1054	CCAAT/enhancer binding protein (C/EBP), gamma (CEBPG)	-6.1/-4.6	-4.0/-1.5
38474_at	875	Cystathionine-beta-synthase (CBS)	-2.5/-1.9	-4.5/-3.0
40074_at	10797	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2 (MTHFD2)	-5.9/-5.4	-4.0/-1.7
37188_at	5106	Phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PCK2)	-4.4/-2.6	-7.3/-5.6
34367_at	26227	Phosphoglycerate dehydrogenase (PHGDH)	-1.3/-1.3	-2.8/-1.5
36178_at	6472	Serine hydroxymethyltransferase 2 (mitochondrial) (SHMT2)	-2.3/-1.6	-2.5/-1.5
40782_at	9249	Dehydrogenase/reductase (SDR family) member 3 (DHRS3)	-4.1/-3.4	-1.5/-1.5
40408_at	833	Cysteinyl-tRNA synthetase (CARS)	-2.8/-2.2	-2.2/-1.1
39342_at	4141	Methionine-tRNA synthetase (MARS)	-2.5/-1.9	-2.7/-1.5
38473_at	6897	Threonyl-tRNA synthetase (TARS)	-2.5/-1.9	-2.1/-1.2
38977_at	8565	Tyrosyl-tRNA synthetase (YARS)	-3.1/-2.3	-2.3/-1.1
		Others		
39333_at	1282	Collagen, type IV, α 1 (COL4A1)	-2.7/-2.1	-1.2/-1.1
37892_at	1301	Collagen, type XI, α 1 (COL11A1)	-2.5/-1.6	-1.2/-1.2
41419_at	51454	GULP, engulfment adaptor PTB domain containing 1 (GULP1)	-2.6/-2.0	-1.3/1.3
39695_at	1604	CD55 molecule, decay accelerating factor for complement (CD55)	-2.2/-1.7	-1.2/1.2
36629_at	1931	TSC22 domain family 3 (TSC22D3)	-2.6/-1.7	-1.1/-1.3
38356_at	10468	Follistatin (FST)	-1.9/-1.9	-1.5/-1.3

33682_at	2825	G protein-coupled receptor 1 (GPR1)	-2.2/-1.9	-1.6/1.3
35785_at	23710	GABA(A) receptor-associated protein like 1 (GABARAPL1)	-2.2/-1.8	-1.2/1.1
37615_at	2887	Growth factor receptor-bound protein 10 (GRB10)	-3.2/-2.8	-1.9/-1.5
37032_at	4837	Nicotinamide N-methyltransferase (NNMT)	-2.5/-1.8	-1.3/1.0
38754_at	26471	Nuclear protein 1 (NUPR1)	-2.3/-1.9	-2.5/-1.5
40689_at	6400	Sel-1 suppressor of lin-12-like (*C. elegans*) (SEL1L)	-1.7/-1.6	-1.9/-2.0
41778_at	6510	Solute carrier family 1 (neutral amino acid transporter), member 5 (SLC1A5)	-2.6/-2.0	-2.5/-1.8
		Unknown function		
36562_at	9811	KIAA0427 (KIAA0427)	-2.3/-1.6	-1.1/1.0
36070_at	57214	KIAA1199 (KIAA1199)	-2.4/-1.6	1.2/1.2

Probe set ID according to Affymetrix. Gene ID, name and official symbol according to Entrez Gene.

ii) Real-time quantitative RT-PCR validation of gene expression changes

The high sensitivity of DNA microarray technology can yield false positive results, but the high costs of the analysis limit the chances of replicating experiments as many times as desired. Therefore, results are usually validated by an independent technique. Most researchers currently validate microarray experiments by confirming the expression of some of the genes with altered expression using real-time RT-PCR, given its sensitivity and flexibility. In the present work, real-time RT-PCR was employed to validate the ability of AA to stimulate the expression of 3 genes involved in lipid metabolism (HMGCR, LDLR and ACAT2) and 2 genes involved in cell cycle progression through G_2/M (CCNB1 and TOP2A). The ability of AA to down-regulate the expression of TFRC and tyrosyl-tRNA synthetase (YARS) was also investigated. The expression of these genes was normalised against that of an endogenous control gene, HPRT1. Although the microarray results were obtained using GM969 HDFs, the subsequent real-time RT-PCR validation of microarray results was performed with GM5399 HDFs, which were available in higher number and at a lower passage number.

RNA integrity was determined by denaturing gel electrophoresis. The RNA samples used in this study were of good quality, as indicated by the presence of distinct bands corresponding to the intact 28S and 18S rRNA subunits and the absence of low

Table 6.4. Biological function enrichment in the list of genes down-regulated by AA

Biological function categories	Study categories hits	Study gene list	Total number of genes in category	Total number of genes analysed	Significance
Cellular biosynthesis[1]	11	30	686	8404	P<0.000001
Carboxylic acid metabolism[2]	12	30	318	8404	P<0.000001
Amino acid metabolism[3]	9	30	165	8404	P<0.000001
tRNA aminoacylation[4]	4	30	27	8404	P<0.000001

[1], PHGDH, ASNS, PCK2, TARS, CBS, YARS, CEBPG, MARS, MTHFD2, CARS, ATF4. [2], PHGDH, SHMT2, ASNS, PCK2, TARS, CBS, YARS, MARS, MTHFD2, CARS, DHRS3, ATF4. [3], PHGDH, SHMT2, ASNS, TARS, CBS, YARS, MARS, CARS, ATF4. [4], TARS, YARS, MARS, CARS.

molecular mass degradation products (Figure 6.9). Primers were designed for the specific amplification of HMGCR, LDLR, ACAT2, CCNB1, TOP2A and YARS. The amplification curves, standard curves and dissociation curves corresponding to the optimised amplification conditions for each gene are shown (Figures 6.10-6.15). Standard curves generated by linear regression analysis of log standard DNA *versus* Ct were used to calculate reaction efficiency and to estimate target quantity for each individual sample, as described in Sections 2.11.5 and 5.1.2. R^2 values indicating 'goodness of fit' of the regression line ranged between 0.997-1.000. The amplification efficiencies of the standards ranged between 97.0-99.1 %. The dissociation curves of the amplification products showed a single peak, confirming the specificity of the reaction and the absence of primer dimers. Further confirmation of the reaction specificity was obtained by analysis of the amplification products by gel electrophoresis, which showed a single band with the expected size (Figure 6.16). The amplification conditions used in the amplification of TFRC and HPRT1 targets were as described in Section 5.3.2.

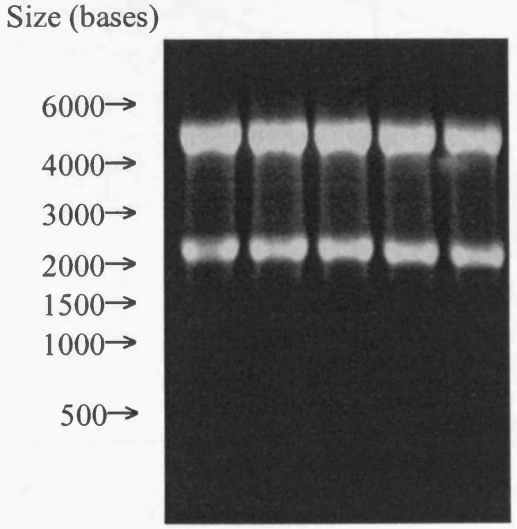


Figure 6.9. Size distribution of T-RNA samples. T-RNA extracted from cells was analysed by denaturing gel electrophoresis as described in Materials and Methods. The example shown is representative of all the samples used in this study.

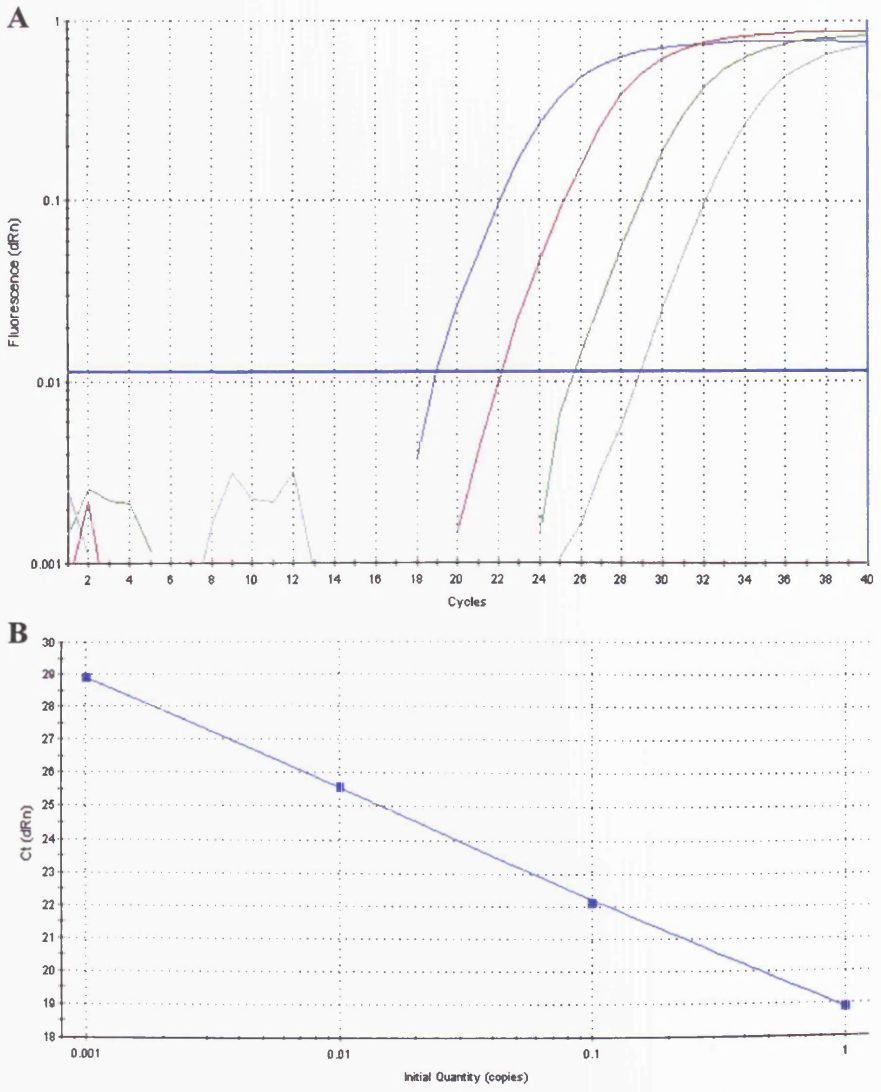


Figure 6.10. Optimized conditions for real-time quantitative RT-PCR analysis of HMGCR transcripts. Amplification was performed as described under Materials and Methods. Annealing temperature was 57 °C, $MgCl_2$ concentration was 3 mM and primer concentration was 200 nM. A, Amplification of a 1:10 serial dilution of standard cDNA. B, Linear regression analysis of log standard cDNA *versus* Ct (R^2 = 1.000, reaction efficiency = 99 %). C, Dissociation curves of multiple PCR products. The existence of a single melting point (Tm = 82.5 °C) indicates the presence of a single amplification product.

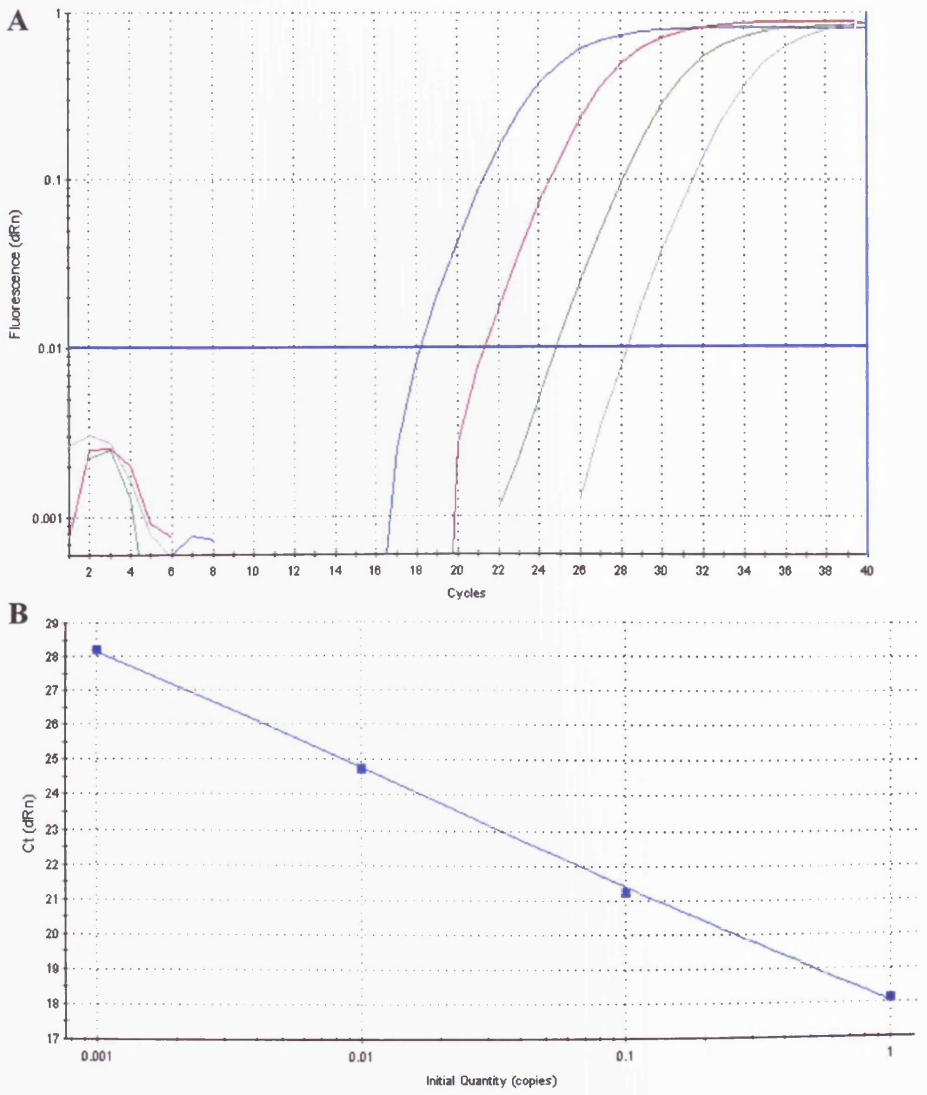


Figure 6.11. Optimized conditions for real-time quantitative RT-PCR analysis of LDLR transcripts. Amplification was performed as described under Materials and Methods. Annealing temperature was 57 °C, $MgCl_2$ concentration was 3 mM and primer concentration was 200 nM. A, Amplification of a 1:10 serial dilution of standard cDNA. B, Linear regression analysis of log standard cDNA *versus* Ct (R^2 = 0.999, reaction efficiency = 97.8 %). C, Dissociation curves of multiple PCR products. The existence of a single melting point (Tm = 83.5 °C) indicates the presence of a single amplification product.

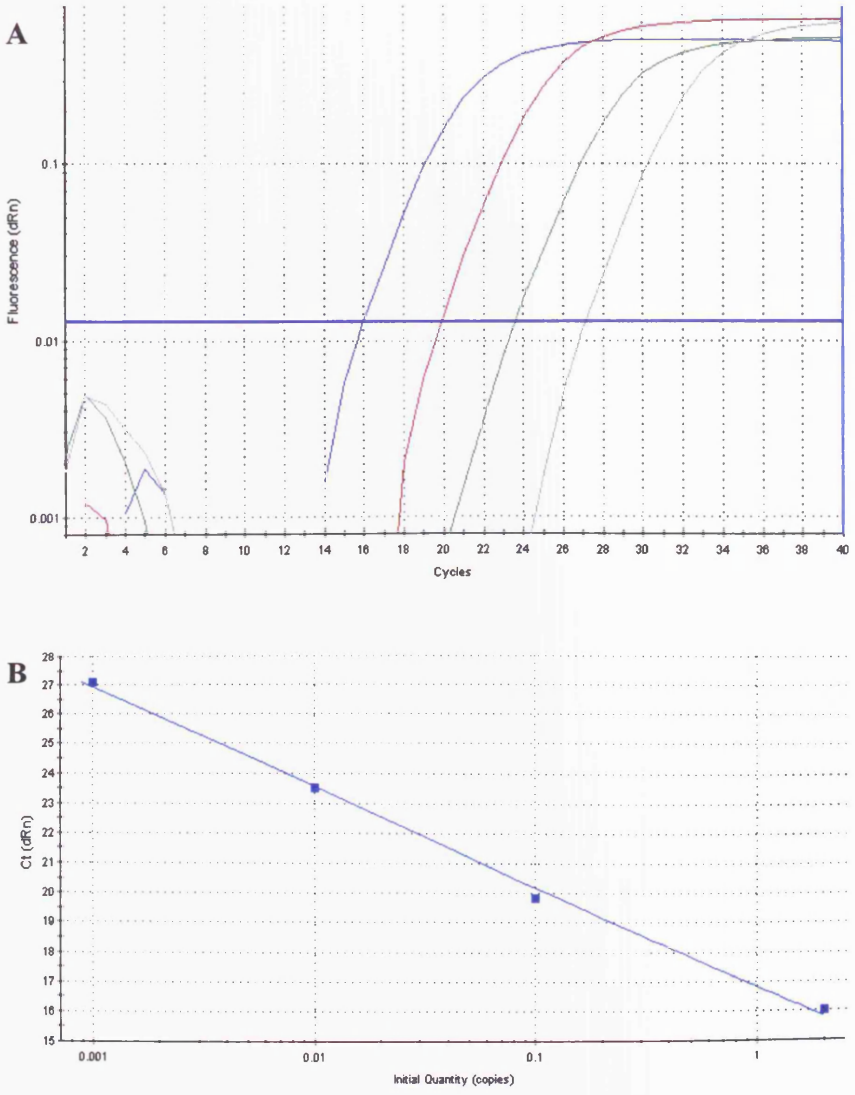


Figure 6.12. Optimized conditions for real-time quantitative RT-PCR analysis of ACAT2 transcripts. Amplification was performed as described under Materials and Methods. Annealing temperature was 57 °C, $MgCl_2$ concentration was 3 mM and primer concentration was 200 nM. A, Amplification of a 1:10 serial dilution of standard cDNA. B, Linear regression analysis of log standard cDNA *versus* Ct (R^2 = 0.997, reaction efficiency = 97.6 %). C, Dissociation curves of multiple PCR products. The existence of a single melting point (Tm = 82 °C) indicates the presence of a single amplification product.

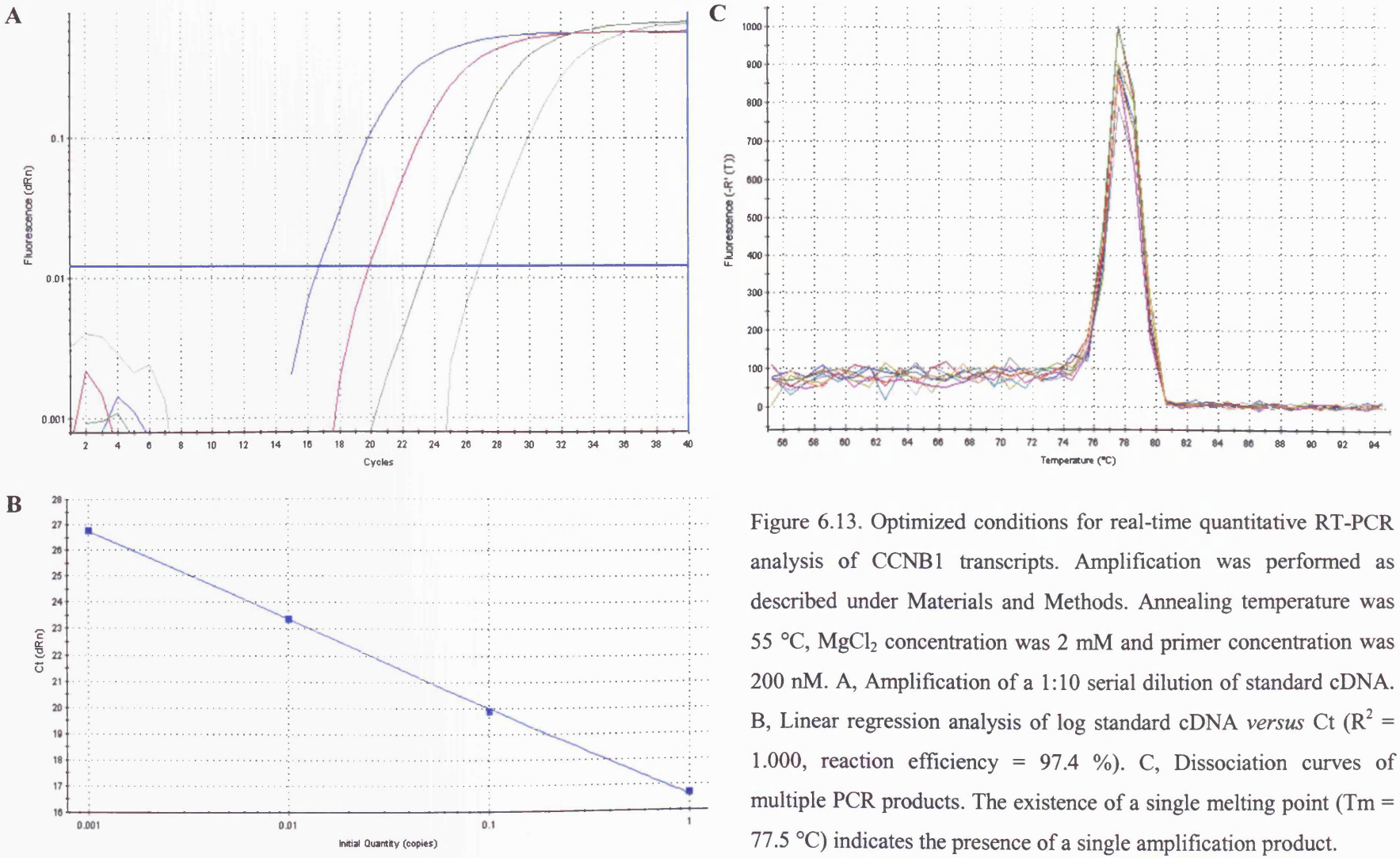


Figure 6.13. Optimized conditions for real-time quantitative RT-PCR analysis of CCNB1 transcripts. Amplification was performed as described under Materials and Methods. Annealing temperature was 55 °C, $MgCl_2$ concentration was 2 mM and primer concentration was 200 nM. A, Amplification of a 1:10 serial dilution of standard cDNA. B, Linear regression analysis of log standard cDNA *versus* Ct (R^2 = 1.000, reaction efficiency = 97.4 %). C, Dissociation curves of multiple PCR products. The existence of a single melting point (Tm = 77.5 °C) indicates the presence of a single amplification product.

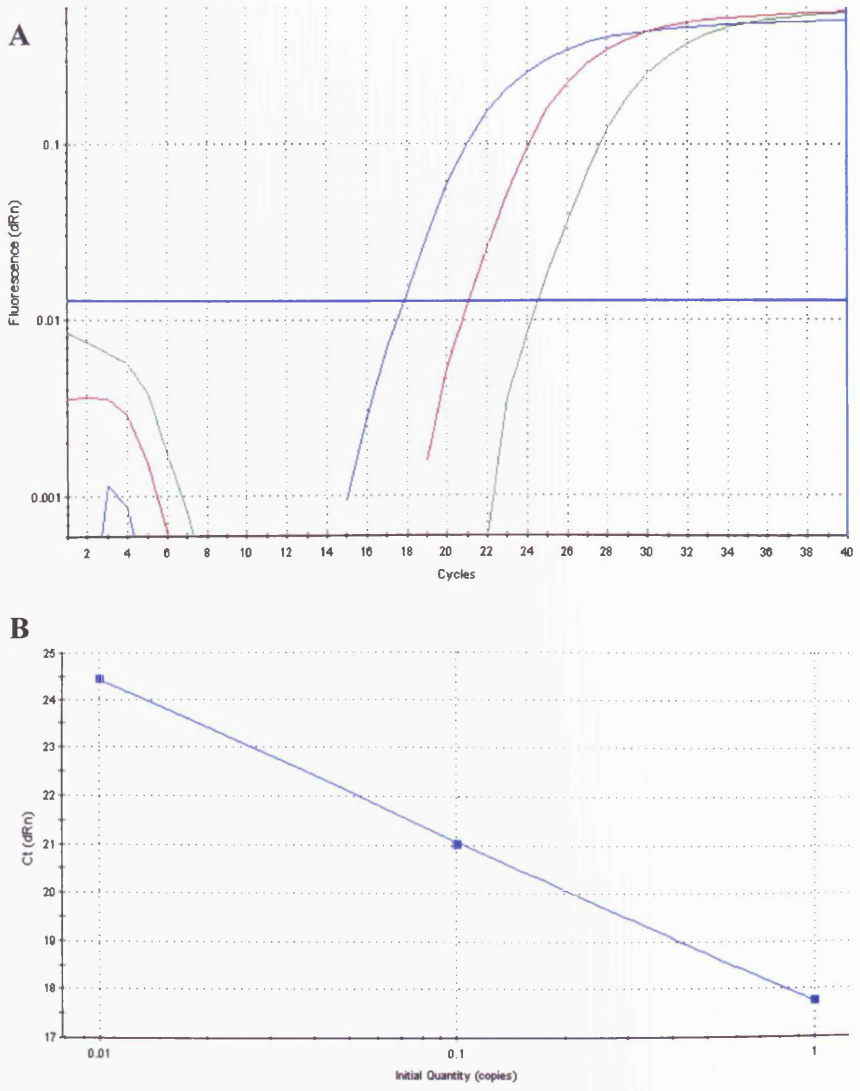
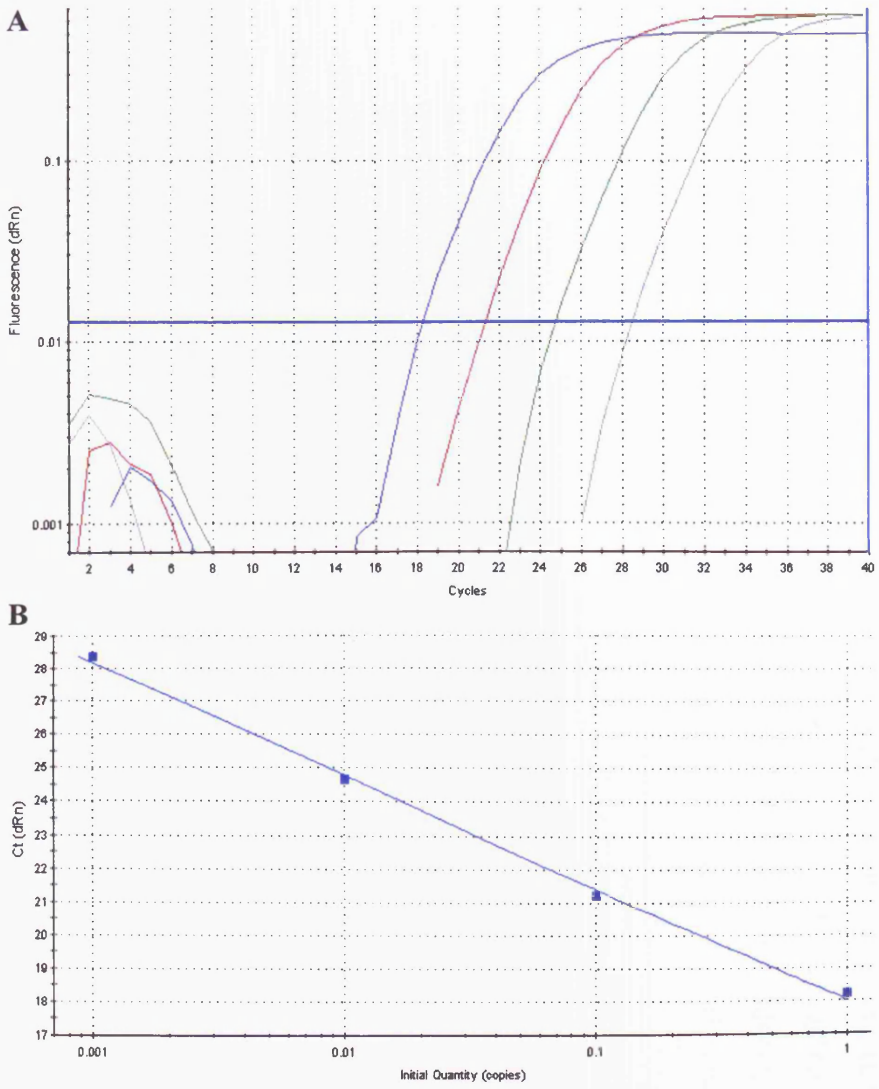


Figure 6.14. Optimized conditions for real-time quantitative RT-PCR analysis of TOP2A transcripts. Amplification was performed as described under Materials and Methods. Annealing temperature was 55 °C, $MgCl_2$ concentration was 2 mM and primer concentration was 200 nM. A, Amplification of a 1:10 serial dilution of standard cDNA. B, Linear regression analysis of log standard cDNA *versus* Ct (R^2 = 1.000, reaction efficiency = 99.1 %). C, Dissociation curves of multiple PCR products. The existence of a single melting point (Tm = 78.5 °C) indicates the presence of a single amplification product.



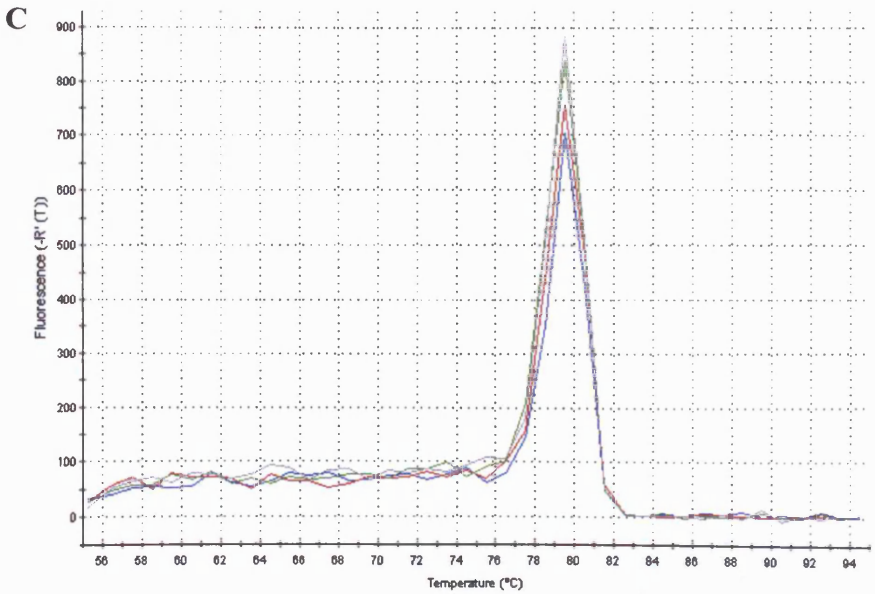


Figure 6.15. Optimized conditions for real-time quantitative RT-PCR analysis of YARS transcripts. Amplification was performed as described under Materials and Methods. Annealing temperature was 55 °C, $MgCl_2$ concentration was 2 mM and primer concentration was 200 nM. A, Amplification of a 1:10 serial dilution of standard cDNA. B, Linear regression analysis of log standard cDNA *versus* Ct (R^2 = 0.998, reaction efficiency = 97 %). C, Dissociation curves of multiple PCR products. The existence of a single melting point (Tm = 79.5 °C) indicates the presence of a single amplification product.

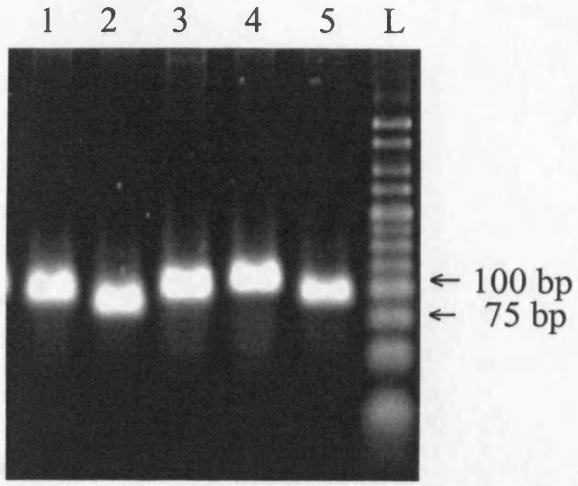


Figure 6.16. Analysis of RT-PCR amplification products by gel electrophoresis. An aliquot of PCR product was examined on a 3 % agarose gel. Electrophoresis was run as described in Materials and Methods. PCR products were visualised under UV after EtBr staining. 1, HMGCR; 2, LDLR; 3, ACAT2; 4, CCNB1; 5, TOP2A; L, DNA HyperLadder V.

The effect of 100 μM AA or AA2P on the steady-state levels of the mRNA of the 6 genes mentioned above (YARS, TFRC, LDLR, HMGCR, ACAT2, CCNB1 and TOP2A) in serum starved GM5399 HDFs was assessed by real-time RT-PCR, using HPRT1 as an endogenous control gene. According to the microarray experiments, YARS mRNA was down-regulated at 8 and 24 hours of exposure to AA (Table 6.2). Real-time RT-PCR measurement of YARS mRNA levels in serum starved HDFs exposed to 100 μM AA for 8 or 24 hours confirmed the same trend of down-regulation (Figure 6.17). The ability of AA to decrease the steady-state levels of TFRC mRNA in HDFs incubated in complete growth medium was described in Section 5.2.2. This was accompanied by a significant reduction of the number of receptors at the cell-surface. Results presented in Figure 6.18 show that AA and AA2P reduce the steady-state levels of TFRC mRNA in serum starved HDFs.

The effects of AA or AA2P on the expression of LDLR, HMGCR and ACAT2 were also investigated. These genes were selected from a set of 12 lipid metabolism genes that were up-regulated by AA at the 8 hours and 24 hours time points in the microarray experiments. The up-regulation of all three genes at an intermediate time point (12 hours) was confirmed by real-time RT-PCR analysis (Figure 6.19). The expression of 2 G_2/M phase genes (CCNB1 and TOP2A) that were up-regulated by AA at the 24 hours time point in the microarray experiments was also validated by real-time RT-PCR analysis (Figure 6.20).

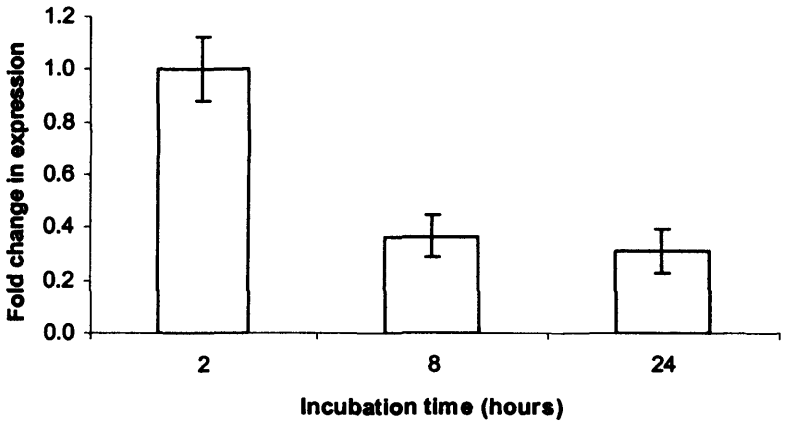


Figure 6.17. Effect of AA on YARS mRNA expression in serum starved HDFs. GM5399 HDFs were incubated in low-serum medium for 48 hours and further incubated with low-serum medium alone or in the presence of 100 μM AA or AA2P for 8 or 24 hours. T-RNA was extracted and the steady-state levels of YARS mRNA measured by real-time RT-PCR. Results are the mean ± S.D. from 3 separate determinations.

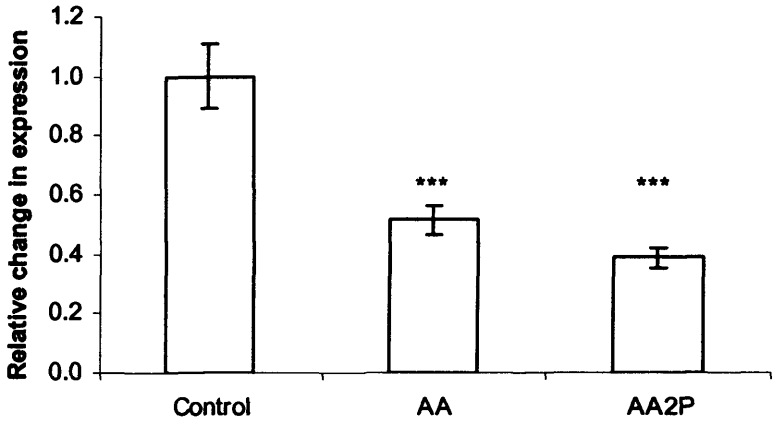


Figure 6.18. Effect of vitamin C on TFRC mRNA expression in serum starved HDFs. GM5399 HDFs were incubated in low-serum medium for 48 hours and further incubated with low-serum medium alone or in the presence of 100 μM AA or AA2P for 12 hours. T-RNA was extracted and the steady-state levels of TFRC mRNA measured by real-time RT-PCR. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* control.

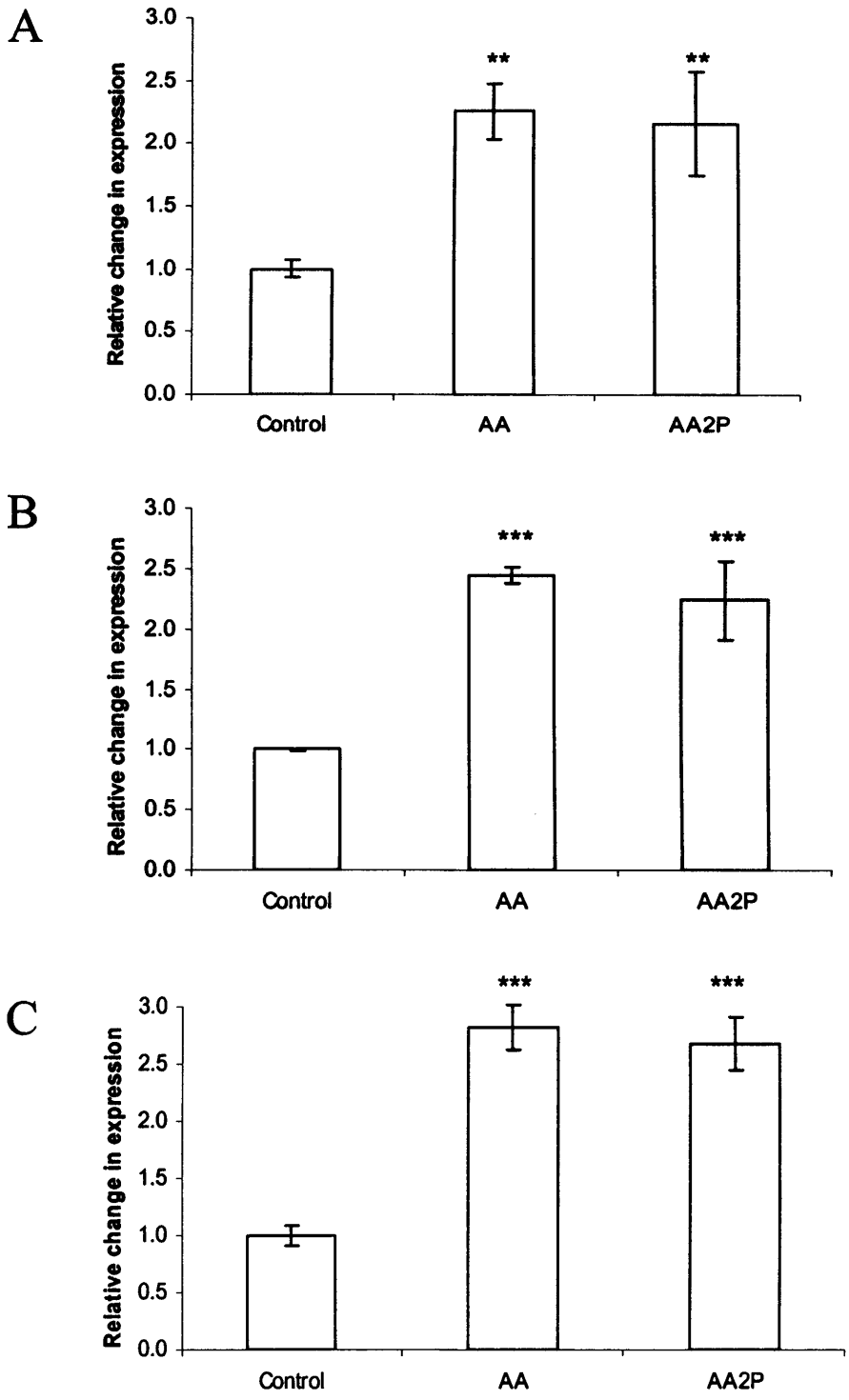


Figure 6.19. Effect of vitamin C on mRNA expression of lipid metabolism genes in serum starved HDFs. GM5399 HDFs were incubated in low-serum medium for 48 hours and further incubated with low-serum medium alone or in the presence of 100 μM AA or AA2P for 12 hours. T-RNA was extracted and the steady-state levels of LDLR (A), HMGCR (B) and ACAT2 (C) mRNA measured by real-time RT-PCR. Results are the mean ± S.D. from 3 separate determinations. **, $P < 0.005$ *versus* control; ***, $P < 0.001$ *versus* control.

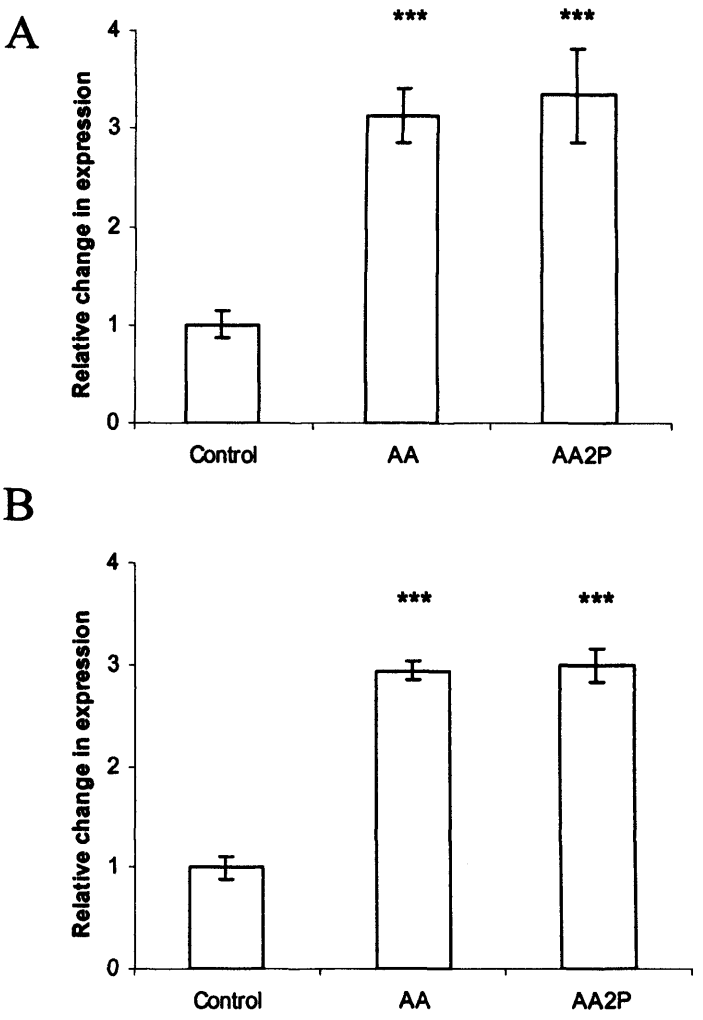


Figure 6.20. Effect of vitamin C on mRNA expression of G_2/M phase genes in serum starved HDFs. GM5399 HDFs were incubated in low-serum medium for 48 hours and further incubated with low-serum medium alone or in the presence of 100 μM AA or AA2P for 24 hours. T-RNA was extracted and the steady-state levels of CCNB1 (A) and TOP2A (B) mRNA measured by real-time RT-PCR. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* control.

6.4.4. Effects of vitamin C in contact-inhibited fibroblasts

The experiments described above illustrated the effects of a single dose of AA in non-dividing populations of HDFs that were kept in a G_0/G_1 cell cycle arrest by serum starvation. These effects include the up-regulation of genes associated with G_2/M phase, suggesting that AA may promote cell cycle progression in normal cells. So, it would be interesting to investigate if AA promotes cell division in HDFs, which would require using cells that are not deprived of serum. Likewise, it would be useful to determine if the effects of AA described above are equally relevant in cells that are not deprived of

serum constituents. HDFs grown to a post-confluent state, despite being kept in growth medium, are not undergoing a cell division rate as high as that of sub-confluent cells, so they may represent more closely the growth characteristics of these cells *in vivo* than exponentially growing or serum starved cells. It would also be useful to investigate if the effects of AA studied above are the result of continued exposure to AA over time, rather than just the effect of a single dose. So, a system was adopted where post-confluent HDFs were continuously supplemented with 100 μM AA or AA2P added fresh every day for a period of 5 days. These experiments were undertaken with GM5659 HDFs. As discussed in Chapter III, the exposure of HDFs to AA at post-confluent density is expected to represent a way to give AA to cells without causing a cytotoxic effect. In fact, when GM5659 cells were grown to confluence and then incubated with 20-500 μM AA or AA2P added fresh everyday for fours days, cell viability was not significantly different from that of control cells and remained > 96 %, as judged by their ability to exclude trypan blue (Table 6.5). This confirmed that vitamin C is not cytotoxic to post-confluent GM5659 HDFs. In addition, these cells are able to take up AA from the medium in a time-dependent manner, with intracellular AA concentration reaching a plateau between 12 and 24 hours of incubation (Section 4.2.1). Therefore, adding 100 μM AA or AA2P at 24-hour intervals should maintain a constant level of intracellular AA repletion.

Visual inspection revealed that, after 5 days of incubation in complete growth medium, control HDFs remained a tightly packed, 2-dimensional monolayer, with only minimal cell overlap. It can therefore be concluded that, in these cells, growth was limited by contact inhibition. In contrast, HDFs incubated with AA or AA2P exhibited post-confluent growth, as indicated by a much higher cell density and extensive cell overlap (Figure 6.21). It can thus be concluded that AA stimulates post-confluent growth in situations where cell proliferation is limited by contact inhibition.

The effects of AA or AA2P on the steady-state levels of the mRNA of iron metabolism (TFRC), lipid metabolism (LDLR, HMGCR and ACAT2) and G_2/M phase (CCNB1 and TOP2A) genes was also investigated in post-confluent GM5659 HDFs continuously exposed to AA or AA2P added fresh everyday for 5 days. As for nearly confluent GM5399 HDFs (Section 5.2.2) and serum starved GM969 and GM5399 HDFs (Section 6.2.3) incubated with vitamin C for 12 hours, continued exposure to AA and AA2P significantly reduced TFRC mRNA expression in post-confluent GM5659

Table 6.5. Effect of 5-day AA or AA2P supplementation on the viability of post-confluent GM5659 HDFs

		% Viability
AA	0 μM	98.2 ± 1.3
	20 μM	97.4 ± 0.6
	100 μM	96.6 ± 0.7
	500 μM	98.8 ± 0.3
AA2P	20 μM	97.3 ± 0.5
	100 μM	96.9 ± 2.3
	500 μM	97.7 ± 0.9

Results are the mean ± S.D. from 3 separate determinations.

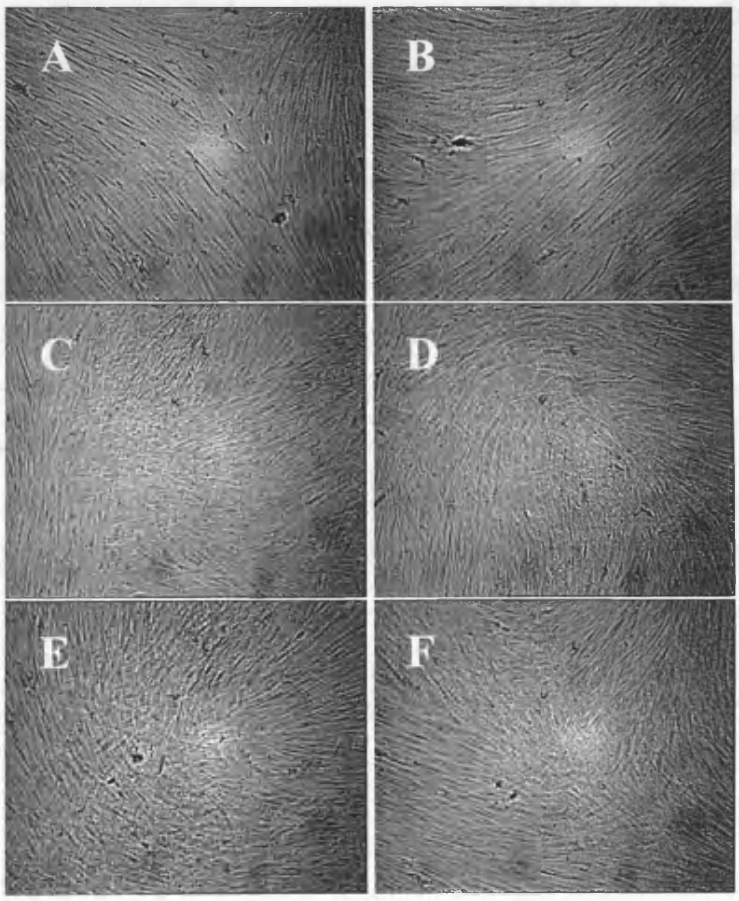


Figure 6.21. Effect of vitamin C on post-confluent growth of HDFs. GM5659 HDFs were grown until becoming a confluent monolayer and then further incubated for 5 days with growth medium alone (A, B) or in the presence of 100 μM AA (C, D) or AA2P (E, F) freshly added every day. Images were captured in a light microscope with 100 × magnification.

cells (Figure 6.22). Continued supplementation with AA or AA2P also caused significant increases in the expression of lipid metabolism genes (Figure 6.23).

Although the fold changes of expression of HMGCR and ACAT2 were lower than those observed in serum starved cells, the LDLR mRNA was up-regulated to a higher extent in post-confluent GM5659 HDFs. The expression of the G_2/M genes CCNB1 and TOP2A was also significantly up-regulated by continuous AA or AA2P supplementation in these cells, to a similar extent to the increase caused by single dose AA or AA2P in serum starved HDFs (*i.e.* 3-fold increase) (Figure 6.24). The latter observation and the fact that AA and AA2P stimulate the post-confluent growth of contact-inhibited HDFs suggest that AA modulates cell cycle progression in these cells. The cell cycle distribution of control and vitamin C-supplemented HDFs during the period of post-confluent incubation was assessed by FACS. As described before, cell proliferation is limited when GM5659 HDFs reach confluence due to contact inhibition. Despite the fact that cells are continuously exposed to growth factors derived from the FBS present in the medium, continued incubation resulted in a clear, time-dependent increase in the proportion of cells arrested at the G_0/G_1 phase the cell cycle (Figure 6.25). Naturally, this was accompanied by a dramatic decrease in the proportion of cells undergoing DNA synthesis (*i.e.* in the S phase of the cell cycle). Notably, a significantly higher number of cells in the S phase of the cell cycle was observed in HDFs continuously exposed to AA or AA2P (Table 6.6). At the end of 3 and 5 days of post-confluent growth, the percentage of cells undergoing DNA synthesis was 2-fold and 3-fold higher, respectively, in HDFs incubated with the two vitamin C derivatives.

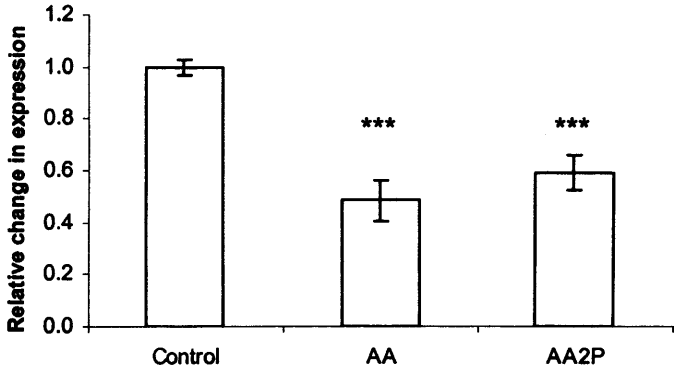


Figure 6.22. Effect of vitamin C on mRNA expression of TFRC in post-confluent HDFs. Confluent GM5659 HDFs were incubated in complete growth medium alone or in the presence of 100 μM AA or AA2P added fresh everyday for 5 days. T-RNA was extracted and the steady-state levels of TFRC mRNA measured by real-time RT-PCR. Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* control.

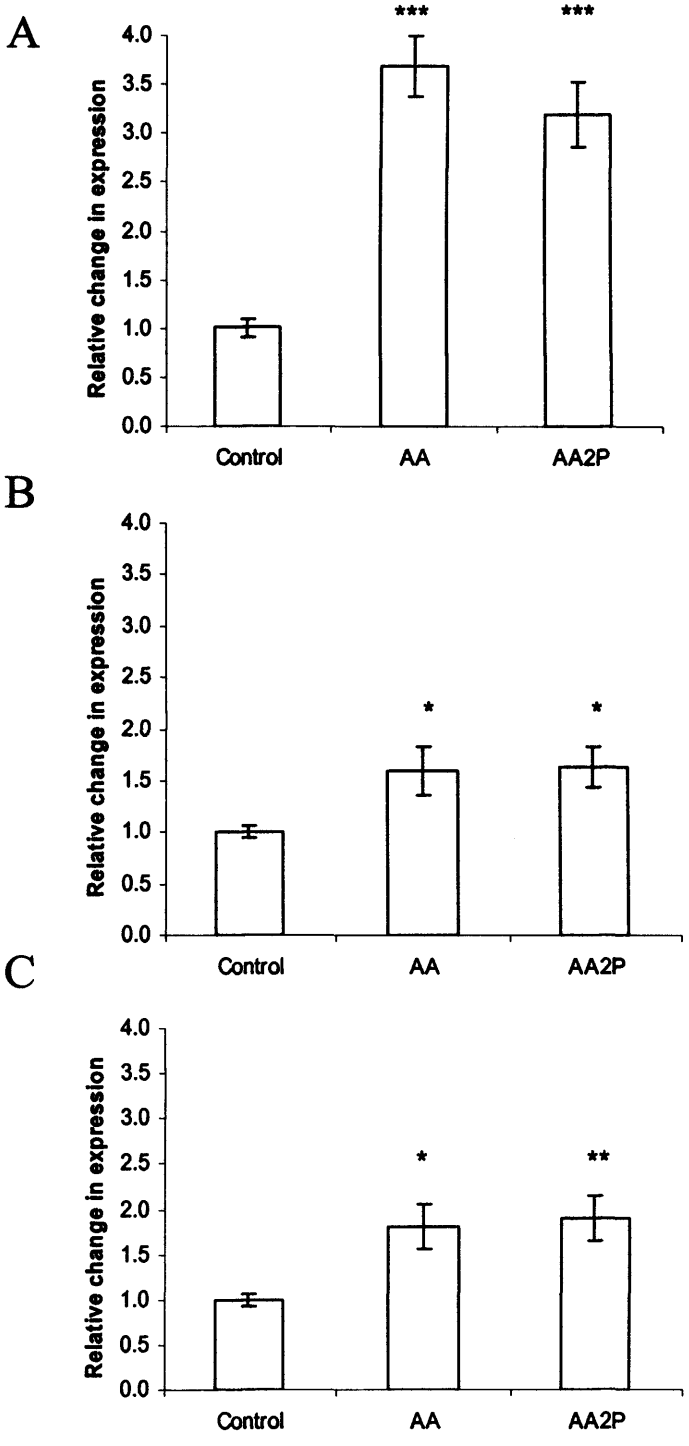


Figure 6.23. Effect of vitamin C on mRNA expression of lipid metabolism genes in post-confluent HDFs. Confluent GM5659 HDFs were incubated in complete growth medium alone or in the presence of 100 μM AA or AA2P added fresh everyday for 5 days. T-RNA was extracted and the steady-state levels of LDLR (A), HMGCR (B) and ACAT2 (C) mRNA measured by real-time RT-PCR. Results are the mean ± S.D. from 3 separate determinations. *, $P < 0.05$ *versus* control; **, $P < 0.005$ *versus* control; ***, $P < 0.001$ *versus* control.

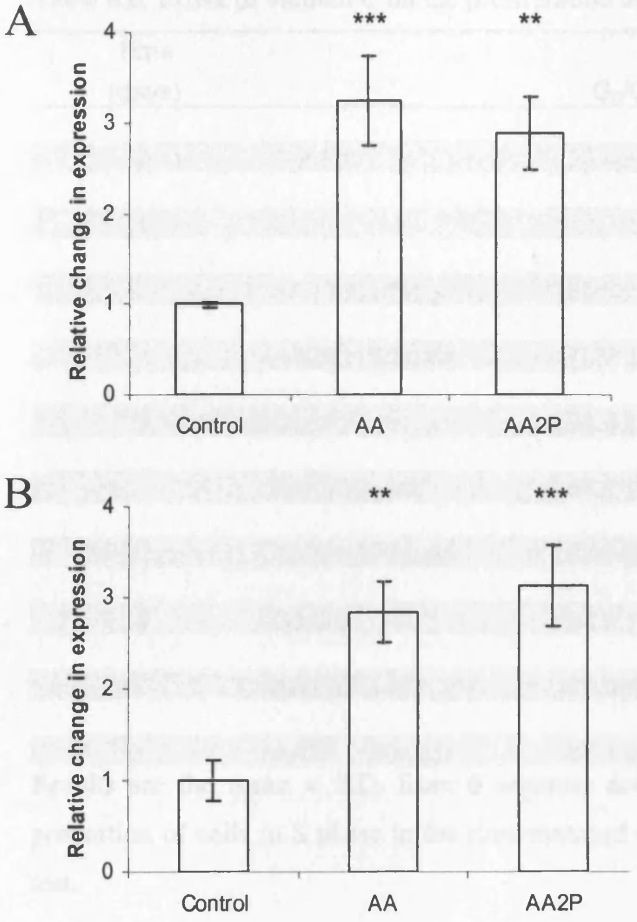


Figure 6.24. Effect of vitamin C on mRNA expression of G_2/M phase genes in post-confluent HDFs. Confluent GM5659 HDFs were incubated in complete growth medium alone or in the presence of 100 μM AA or AA2P added fresh everyday for 5 days. T-RNA was extracted and the steady-state levels of CCNB1 (A) and TOP2A (B) mRNA measured by real-time RT-PCR. Results are the mean ± S.D. from 3 separate determinations. **, $P < 0.005$ *versus* control; ***, $P < 0.001$ *versus* control.

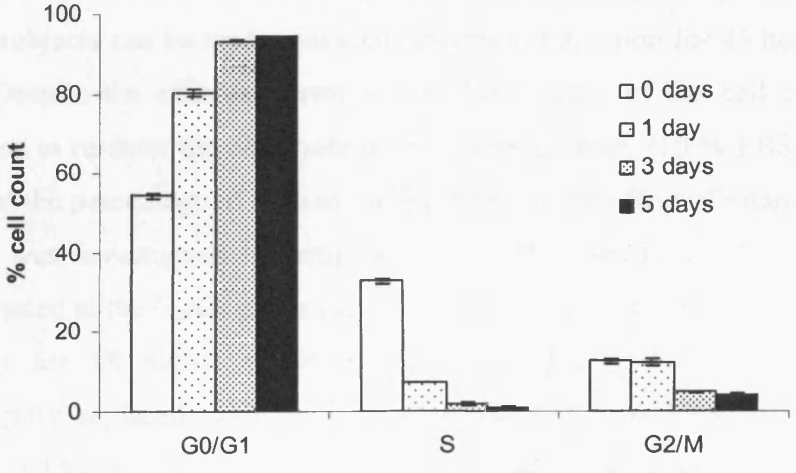


Figure 6.25. Effect of contact inhibition on cell cycle distribution in HDFs. After reaching confluence, GM5659 HDFs were incubated in complete growth medium added fresh everyday for 5 days. Cell cycle distribution was determined by FACS. Results are the mean ± S.D. from 3 separate determinations.

Table 6.6. Effect of vitamin C on the proliferation of contact-inhibited GM5659 HDFs

Time (days)		Cell cycle distribution		
		G_0/G_1	S	G_2/M
0	Control	54.4 ± 0.6	32.9 ± 0.8	12.7 ± 0.8
1	Control	83.2 ± 3.7	6.0 ±1.9	10.8 ± 1.9
	AA (100 μM)	75.3 ± 0.6	10.7 ± 0.9 **	14.1 ± 0.6
	AA2P (100 μM)	75.2 ± 0.9	10.4 ± 0.5 **	14.5 ± 0.5
3	Control	91.4 ± 2.1	2.8 ± 1.1	5.9 ± 1.1
	AA (100 μM)	87.1 ± 3.0	6.0 ± 1.7 **	6.9 ± 1.4
	AA2P (100 μM)	87.5 ± 2.7	6.2 ± 1.6 **	6.3 ± 1.1
5	Control	93.9 ± 0.8	1.8 ± 0.7	4.3 ± 0.1
	AA (100 μM)	88.5 ± 0.6	5.2 ± 1.0 **	6.3 ± 0.5
	AA2P (100 μM)	88.4 ± 0.6	5.5 ± 0.7 **	6.2 ± 0.6

Results are the mean ± S.D. from 6 separate determinations. **, P = 0.0051 *versus* the proportion of cells in S phase in the time-matched control, as determined by Mann Whitney test.

Finally, experiments were carried out to further investigate if AA modulates cell cycle re-entry in HDFs. A previous study showed that primary HDFs obtained from healthy subjects can be made quiescent by serum starvation for 48 hours (Danesh *et al.* 1999). Despite the efficient arrest at the G_0/G_1 phase of the cell cycle, HDFs were stimulated to re-enter the cell cycle by exposure to serum (10 % FBS), which naturally increased the percentage of cells in the S phase. So, the effect of vitamin C on cell cycle re-entry was investigated in serum starved HDFs. Nearly confluent GM5659 HDFs were arrested at the G_0/G_1 phase the cell cycle by incubation in low-serum medium (0.5 % FBS) for 48 hours, as described before (Figure 6.2). Culture medium was subsequently replaced with low-serum medium (0.5 % FBS) or complete growth medium (10 % FBS) and cells were further incubated for 24 hours. As depicted in Table 6.7, cells that were returned to low-serum medium for further 24 hours remained arrested at G_0/G_1. In contrast, a proportion of the cells that were returned to complete growth medium re-entered the cell cycle, as indicated by an increase in the percentage of cells in S and G_2/M phases. The addition of AA or AA2P with low-serum medium

Table 6.7. Effect of vitamin C on cell cycle re-entry in serum starved GM5659 HDFs

Time post serum starvation	Cell cycle distribution		
	G_0/G_1	S	G_2/M
0 hours	96.2 ± 0.2	0.6 ± 0.0	3.3 ± 0.2
24 hours low-serum medium	95.8 ± 0.2	0.8 ± 0.2	3.5 ± 0.1
24 hours low-serum medium + AA (100 µM)	94.0 ± 0.4	1.0 ± 0.0	5.0 ± 0.3
24 hours low-serum medium + AA2P (100 µM)	94.2 ± 0.2	1.0 ± 0.1	4.8 ± 0.1
24 hours growth medium	87.5 ± 0.3	6.0 ± 0.4	6.6 ± 0.1
24 hours growth medium + AA (100 µM)	79.0 ± 0.9	12.7 ± 1.0 ***	8.3 ± 0.2
24 hours growth medium + AA2P (100 µM)	79.1 ± 0.8	12.7 ± 0.7 ***	8.2 ± 0.2

Results are the mean ± S.D. from 3 separate determinations. ***, $P < 0.001$ *versus* the proportion of cells in S phase in cells returned to growth medium for 24 hours.

did not change cell cycle arrest at G_0/G_1. But the addition of AA or AA2P to growth medium increased the number of cells undergoing division. In particular, AA and AA2P caused a significant, 2-fold increase in the number of cells undergoing DNA synthesis.

6.5. Discussion

The present work was the first to study the effects of AA on gene expression profiling in normal human cells. Non-dividing populations of HDFs were employed here to allow the study of gene expression in a way that is believed to be more closely related to the *in vivo* situation than actively growing cells (see Section 6.2). This is supported by the work of other authors. For example, despite the fact that fibroblasts are the cell type responsible for most of apolipoprotein D (APOD) expression *in vivo*, APOD mRNA is not detected in replicating sparse HDFs in culture. Expression of APOD is, however, found in quiescent confluent HDFs and in serum starved HDFs (Provost *et al.* 1991).

The effects of AA on gene expression were initially studied in serum-starved HDFs using high-density oligonucleotide DNA microarray analysis. Results were subsequently validated by real-time RT-PCR analysis using HPRT1 as a reference gene. As described in Section 5.2, the choice of a reference gene is crucial for the interpretation of gene expression data. HPRT1 has proved to be a suitable endogenous control in gene expression studies of human peripheral blood mononuclear cells (Dheda

et al. 2004) and human neutrophils (Zhang *et al.* 2005). Moreover, it is a suitable reference gene for expression studies of low abundance transcripts (Zhang *et al.* 2005). In this respect, the current work confirmed that HPRT1 was an adequate reference gene (*i.e.* similar range of Ct values, between 16 and 20 cycles) in the study of TFRC, HO-1, CCNB1, TOP2A, HMGCR, ACAT2, LDLR and YARS. In addition, according to the DNA microarray results, the expression of HPRT1 was invariant in HDFs incubated in the presence of AA. In fact, with accurate RNA quantification, similar Ct values are observed between control HDFs and HDFs treated with different agents, such as AA, iron supplementation or chelation, H_2O_2 or UVA, yielding results like those shown in Figure 5.7. Occasional small differences in Ct values could therefore be attributed to differential RNA input with some confidence.

With the gene filtering criteria adopted (see Section 2.9.7), AA induced changes in the expression of 72 genes. The cluster of up-regulated genes was significantly enriched for genes involved in cholesterol/fatty acid synthesis and for genes involved in cell cycle progression into G_2/M phases of the cell cycle. The latter include 3 genes that regulate the transition of G_2 to M phases of the cell cycle (CCNB1, CDC2 and CSK2) and 7 genes encoding chromosome segregation and spindle associated proteins (TOP2A, GINS1, BUB1B, TPX2, MAPRE2, SMC4 and KNTC2). Genes that regulate and effect chromosome segregation are also known to be co-ordinately up-regulated during M phase in HDFs (Cho *et al.* 2001). Cyclin function is determined by cyclin levels, which are known to oscillate during the different phases of the cell cycle. In mammals, sequential, periodic oscillations in the level of major cyclins reflect changes in the mRNA levels. For example, CCNB1 mRNA and protein are known to accumulate during G_2 phase and abruptly decline at the end of mitosis (Pines and Hunter 1989). During G_2/M, CCNB1 associates with CDC2 (Pines and Hunter 1989). CDC2 levels are also transiently elevated during G_2 and M (De Souza *et al.* 2000). The CCNB1/CDC2 complex accumulates at the centrosome in late G_2 (De Souza *et al.* 2000). CSK2 mRNA expression is also up-regulated during mitosis in HDFs (Cho *et al.* 2001). TOP2A is a protein required during the final stages of DNA replication and during chromosome disjunction at mitosis. The enzyme facilitates chromosome untangling, condensation and segregation during mitosis (Holm *et al.* 1989). Accordingly, the levels of TOP2A mRNA increase in late S and G_2/M and this correlates with increased synthesis of the protein (Goswami *et al.* 1996).

Notably, many of the genes induced by AA in the present study, including regulators of transition of G_2 to M (CCNB1, CDC2 and CSK2), TOP2A or stanniocalcin (STC1), are also induced 24 hours after serum stimulation of HDFs previously arrested by serum deprivation (Iyer *et al.* 1999). The authors related the serum stimulation of HDFs with their role in the process of wound healing. In this respect, it is worth noting that, simultaneously with the up-regulation of G_2/M genes, AA increased the expression of ITGA4 and CDH18. In fact, Cho *et al.* (2001) reported that HDFs express genes that facilitate cell-to-cell adhesion and perform other roles in the extracellular matrix during M phase. It can thus be speculated that AA, by stimulating quiescent HDFs to re-enter the cell cycle, would contribute to the process of wound healing in human skin.

As mentioned, AA increased the expression of a gene involved in fatty acid synthesis (FASN) and a cluster of genes involved in the cholesterol biosynthesis pathway, including LDLR and HMGCR. The cellular content of cholesterol is the result of the balance between exogenous cholesterol uptake via the LDLR and the endogenous synthesis of cholesterol. HMGCR is the rate-limiting enzyme in cholesterol synthesis. The several genes involved in *de novo* cholesterol synthesis (HMGCR, ACAT2, FDPS, IDI1, SQLE and SC4MOL) and uptake (LDLR), sterol metabolism (INSIG1) and in the biosynthesis of fatty acids (FASN) that were up-regulated by AA in the present study are known direct targets of sterol regulatory element-binding proteins (SREBPs) (Horton *et al.* 2003). SREBPs are transcription factors that, in response to low cellular cholesterol, are released from the membrane of the endoplasmic reticulum and migrate to the nucleus, where they increase the transcription of genes involved in the biosynthesis of fatty acids and in the synthesis and uptake of cholesterol. SREBP-1a up-regulates all SREBP-responsive genes and may be required to ensure that there is a supply of cholesterol and fatty acids during periods of rapid cell division. On the other hand, SREBP-1c preferentially up-regulates fatty acid synthesis genes, whereas SREBP-2 preferentially up-regulates cholesterol synthesis genes and the LDLR (Osborne 2000; Horton *et al.* 2002). The induction of SREBP-responsive genes when cellular cholesterol is low depends on the formation of a complex between SREBP and SREBP cleavage-activating protein (SCAP) and its release from the endoplasmic reticulum (Horton *et al.* 2002). INSIG1 is a transcriptional target of SREBPs and its transcription increases in the absence of sterols, as part of a feedback mechanism. In the

presence of sterols, INSIG1 promotes the retention of the SREBP-SCAP complex in the endoplasmic reticulum, thereby blocking SREBP activation (Janowski 2002).

Free cholesterol and fatty acids are important components of the plasma membrane of mammalian cells. Free cholesterol also serves as a precursor for the synthesis of steroid hormones and bile acids (Goldstein and Brown 1977). Cells that are undergoing mitotic division have a higher demand of free cholesterol and fatty acids than quiescent cells. Free cholesterol is required for the synthesis of new membranes before cell division occurs. In the absence of cholesterol, cells can not undergo cytokinesis, which leads to the formation of polyploid, multinucleated cells (Fernandez *et al.* 2004). In fact, the free cholesterol mass of HDFs is transiently elevated between S phase and mitosis (Fielding *et al.* 1999). This cholesterol can be provided by lipoproteins in the medium or synthesised endogenously. Endogenous synthesis of cholesterol is controlled by the activity of HMGCR. However, cells cultured in the presence of serum exhibit low levels of cholesterol synthesis. In fact, cultured fibroblasts and other non-hepatic cell types express cell surface receptors with high-affinity for the most abundant cholesterol-binding protein in plasma, low-density lipoprotein (LDL). The LDLR is regulated by a feedback mechanism that exists to maintain the level of intracellular cholesterol constant (Goldstein and Brown 1977). When cells are in need of cholesterol, they synthesise the LDLR, which becomes readily available at the cell surface. As a consequence, LDL is taken up from the medium by receptor-mediated endocytosis. Cellular uptake of cholesterol via the LDLR suppresses the endogenous synthesis of cholesterol by reducing the activity of HMGCR. When free cholesterol is in excess, acetyl-CoA:cholesterol acetyltransferase (ACAT) attaches a long-chain fatty acid to free cholesterol to form cholesteryl esters, which can be stored in the cell. Excess free cholesterol also suppresses the synthesis of LDLR.

In HDFs and other cell types, LDLR mRNA and cell-surface expression is induced by several mitogenic agents such as growth factors, cytokines and insulin (Mazzone *et al.* 1989; Hsu *et al.* 1994). The transcriptional induction of LDLR by mitogens is associated with an increase in DNA synthesis (Mazzone *et al.* 1989). Platelet-derived growth factor (PDGF) stimulates LDLR activity in aortic smooth mucle cells (Chait *et al.* 1980) and HDFs (Mazzone *et al.* 1989). The HMGCR activity of HDFs is also stimulated by serum factors, including insulin (Brown *et al.* 1974) and PDGF (Fairbanks *et al.* 1986). In addition, basic fibroblast growth factor enhances ACAT activity in arterial smooth muscle cells (Hsu *et al.* 1994). The activation of

growth in quiescent cells is therefore accompanied by the stimulation of lipoprotein cholesterol internalisation and endogenous *de novo* cholesterol synthesis (Mazzone and Pustelnikas 1990). The link between lipid biosynthesis and growth activation is further supported by the elevated expression of HMGCR, LDLR and FASN in tumour cells (Gueddari *et al.* 1993; Peiretti *et al.* 2004, Yang *et al.* 2003). The stimulation of LDLR (Mazzone *et al.* 1990) and FASN (Swinnen *et al.* 2000) expression by mitogens in tumour cells is mediated by SREBPs. It is possible that AA increased the binding of SREBPs to target genes, but that possibility was not investigated here.

The results discussed so far were obtained with serum starved HDFs, but they were subsequently observed in cells arrested by contact inhibition. It is worth noting that, whilst serum starved HDFs are significantly deprived of serum glucose, insulin and growth factors, that is not the case with cells arrested by contact inhibition. Cells in a post-confluent state are not undergoing a cell division rate as high as that of sub-confluent cells, and hence may represent more closely the growth characteristics of cells *in vivo*, but they are still exposed to standard cell growth conditions. In the present study, the expression of LDLR mRNA was elevated by AA to a slightly higher extent in contact inhibited HDFs than in serum starved cells. On the other hand, the AA-mediated fold increase in HMGCR and ACAT2 expression, although statistically significant, was considerably reduced in contact-inhibited HDFs. This may have been due to an inhibitory effect exerted by serum LDL on the expression of these two genes.

As discussed above, the up-regulation of genes involved in cholesterol and fatty acid biosynthesis observed in the present study may be part of the same program of gene activation that ultimately leads to the re-entrance of quiescent HDFs into the cell cycle when incubated with AA. Although HDFs are subjected to contact-dependent inhibition, cells can be stimulated to re-enter the cell cycle and proliferate by the addition of growth factors, leading to the formation of multiple cell layers. Previous studies have shown that AA (100 μM) promoted post-confluent proliferation and collagen synthesis in primary HDFs (Hata *et al.* 1988; Phillips *et al.* 1994). The fact that AA did not up-regulate the transcription of collagen genes in the present study is not surprising, since the stimulatory action of AA on collagen synthesis occurs through post-transcriptional mechanisms (Chan *et al.* 1990). The stimulatory action of AA on cell proliferation and collagen synthesis is also achieved with the stable derivative AA2P (Hata and Senoo 1989). The ability of vitamin C to stimulate post-confluent growth of HDFs was confirmed in the present study in experiments where cells were

incubated with AA or AA2P freshly added every day for up to 5 days. Furthermore, the present study showed that AA increases the percentage of cells that were stimulated to synthesise DNA after serum stimulation of quiescent HDFs. A similar effect was reported by Carpenter and Cohen (1976), who stimulated quiescent cells with human epidermal growth factor. The effect of AA was, however, dependent on the addition of serum to the culture medium. In fact, despite inducing the transcription of G_2/M genes in serum starved HDFs, AA or AA2P did not induce cell cycle re-entry in cells that were kept in low-serum medium. This suggests that whilst AA favours the mitogenic stimulation of quiescent HDFs, other factors are required for cells to divide.

In addition to inducing gene expression, AA has also repressed the expression of 30 genes. AA down-regulated a gene involved in iron homeostasis, TFRC, which is in agreement with the results presented in Chapter V. In addition, AA reduced the expression of genes involved in carboxylic acid metabolism, amino acid metabolism and tRNA aminoacylation. The most down-regulated gene in the present study was ASNS. ASNS is responsible for the synthesis of asparagines from aspartate and glutamine in most mammalian cells. Transcription of the ASNS gene is induced in response to either amino acid (asparagine, leucine, isoleucine or glutamine) or glucose deprivation. The former is known as the amino acid response, whereas the latter situation corresponds to the endoplasmic reticulum stress response, also known as the unfolded protein response pathway due to the observed accumulation of misfolded proteins (Barbosa-Tessmann *et al.* 1999a). In both cases, the effect is mediated by the binding of C/EBPβ (Siu *et al.* 2001) and ATF4 (Siu *et al.* 2002) to a region in the ASNS promoter, the nutrient-sensing response unit (NSRU). ATF4 mRNA is iself up-regulated in response to amino acid or glucose deprivation (Siu *et al.* 2002).

ASNS mRNA accumulates dramatically during glucose deprivation in HepG2 cultured hepatoma cells and this accumulation is repressed by the addition of other sugar metabolites (2-deoxy-d-glucose, fructose, galactose, mannose and xylitol) (Barbosa-Tessmann *et al.* 1999b). AA is structurally related to glucose and, in fact, DHA enters cells through the glucose transporters (Vera *et al.* 1993; Diplock 1994; Rumsey *et al.* 1997). So it could be speculated that AA would repress ASNS expression by mimicking glucose. However, Barbosa-Tessmann *et al.* (1999b) showed that a glucose analogue, 3OMGlc, which is transported into cells but cannot be phosphorylated by hexokinases, did not repress the induction of ASNS caused by glucose deprivation. This suggests that the signal for ASNS induction/repression is not

free glucose, and that further carbohydrate metabolism (such as phosphorylation to glucose 6-phosphate) may be necessary for ASNS transcriptional regulation.

It is possible that at least some of the expression decreases discussed above may also be part of the mitogenic activation of HDFs. For example, nuclear protein 1 (NUPR1) mRNA expression in mouse embryonic fibroblasts is strongly activated as a consequence of cell growth arrest by serum starvation or contact inhibition (Vasseur *et al.* 2002).

Finally, DNA microarray analysis and real-time RT-PCR measure changes in steady-state transcription of a gene. It should be noted, however, that mRNA levels do not necessarily reflect the levels of protein being synthesised by the cell, as regulation also occurs at post-transcriptional levels. Nevertheless, the induction of an expression profile consistent with cell cycle progression through G_2/M was supported by evidence from flow cytometry experiments and from visual inspection of cell growth. Likewise, down-regulation of TFRC mRNA is supported by a reduction in the number of receptors at the cell surface, as reported in Section 5.4.2.

6.6. Summary

Incubation of serum-starved HDFs with AA (100 μM) induced changes in the expression of 72 genes. The up-regulated genes are involved in the synthesis of cholesterol and fatty acids or in cell cycle progression into G_2/M phases of the cell cycle. The down-regulated genes are involved in cell metabolism and protein synthesis. Results were confirmed using a different cell model, namely HDFs arrested by contact inhibition. Overall, gene expression profiling results show that AA affects normal cellular metabolism by regulating gene expression. In particular, AA induces a mitogenic stimulus in quiescent cells, with concomitant stimulatory effects on lipid metabolism and cell division. Cell cycle analysis studies confirmed that AA stimulates non-dividing cell populations to re-enter the cell cycle and ultimately undergo cell division. Although it remains to be determined if AA can promote fibroblast activation *in vivo*, the present study suggests that AA may stimulate the process of wound healing, where quiescent dermal fibroblasts are required to undergo cell division.

CHAPTER VII

FINAL DISCUSSION

7.1. Vitamin C modulation of oxidative DNA damage as a consequence of altered iron homeostasis

In the human body, cells are constantly exposed to the effects of ROS derived from either external sources or the endogenous metabolism. ROS derived from either source are thought to be involved in the aetiology of a wide variety of diseases, including carcinogenesis, and ageing. The deleterious effects of ROS are countered by the existence of cellular antioxidant defences. Vitamin C is traditionally regarded as one of the most important water-soluble antioxidants in human plasma, where it is thought to scavenge reactive oxygen and nitrogen species and to recycle other antioxidants. Health claims derived from observational epidemiological studies have associated diets rich in antioxidants with reduced risks for certain cancers and cardiovascular disease, increased function of the immune system and a reduction of stress. Based on these claims, consumers' interest for antioxidant supplements has been increasing rapidly. However, data from intervention studies are contradictory and overall there is still not enough evidence to claim that vitamin C supplementation brings additional benefit through the prevention of DNA oxidation in humans consuming adequate amounts of vitamin from their diet. It is possible that the beneficial effects of vitamin C supplementation are only relevant to those individuals with low levels of plasma ascorbate, such as smokers, or in pathological conditions associated with high steady-state levels of oxidative stress. Furthermore, it is possible that in certain conditions vitamin C supplements may have deleterious effects *in vivo*. These effects may, in turn, be related to the ability of AA to enhance the deleterious effects of iron in cells, tissues and organisms. The present work has studied the effects of AA on cellular iron homeostasis in order to establish whether AA may have overall protective or deleterious effects with respect to iron-dependent, ROS-mediated cell injury.

Ferric ammonium citrate (FAC) and desferrioxamine (DFO) are two well-established modulators of cellular iron homeostasis and were employed to demonstrate that, in the HDFs used in the present study, all the iron-related parameters assessed were responsive to iron supplementation and chelation. As expected, supplementation of HDFs with iron (FAC) expanded the intracellular pool of PG SK-chelatable iron, increased cellular ferritin and reduced the expression of TFRC. Conversely, exposure to a specific

iron chelating agent (DFO) reduced intracellular PG SK-chelatable iron and cellular ferritin, and increased the expression of TFRC.

Taking into account the above, the current study presents four lines of evidence that AA affects cellular iron homeostasis. First, AA augmented the intracellular pool of PG SK-chelatable iron, as described in Chapter V. Second, AA increased the levels of cellular ferritin by a post-transcriptional mechanism that is in agreement with its regulation by the IRP/IRE system (Chapter V). This is further supported by the fact that ferritin transcripts were not up-regulated in the microarray experiment, despite the fact that the human genome U95Av2 arrays contain probe sequences for ferritin heavy chain polypeptide (33943_at) and ferritin light polypeptide (35083_at) mRNAs. Third, AA reduced the expression of the TFRC (Chapter V). Although the expression of the TFRC has been associated with cell proliferation (Owen and Kühn 1987), AA and AA2P significantly reduced expression of TFRC in proliferating GM5399 cells, in GM5399 and GM969 cells synchronised at G_0/G_1 phases of the cell cycle by serum starvation and in post-confluent GM5659 HDFs continuously incubated with vitamin C for 5 days. Taken together, these data show that the effect of vitamin C on TFRC expression is independent of the cell proliferating status. Fourth, AA sensitised cells towards iron-dependent, H_2O_2-induced DNA damage and cell death, as described in Chapter IV. Overall, these data support the view that AA expands the intracellular pool of catalytically active iron of HDFs.

In theory, AA could expand the intracellular pool of catalytically active iron by facilitating iron mobilisation from intracellular iron storage sites and/or by increasing cellular uptake of low molecular weight iron complexes. As discussed in Section 5.5.1, it is possible that AA may diffuse into ferritin, reduce ferric to ferrous iron and chelate it, assisting in its diffusion through the ferritin channels (Tufano *et al.* 1981; Boyer and McCleary 1987). AA could also possibly reduce any ferric iron available at the cell-surface and favour the uptake of ferrous iron through the divalent metal transporter (DMT1) into the intracellular LIP. As described in Section 1.3.2, mammalian cells take up iron bound to low molecular weight, low affinity chelators such as ferric citrate or FAC through non-specific membrane transporters (Richardson and Baker 1992; Oshiro *et al.* 1993). Reduction of extracellular Fe^{3+} to Fe^{2+} appears to be required for this process of non-transferrin-bound iron (NTBI) uptake (Randell *et al.* 1994). Addition of AA to the medium

favours radioactive iron uptake from ferric iron chelates (Quail and Morgan 1994; Jordan and Kaplan 1994; Han *et al.* 1995; Parkes *et al.* 1997; May *et al.* 1999), suggesting that AA may be the source of reducing equivalents for cell-surface reduction of ferric iron. Whether this could account for all the effects observed in the present work is, however, not clear. DNA damage experiments performed in serum-free medium showed that the addition of extracellular iron as either FAC or *holo*-transferrin did not increase, but actually reduced the DNA damage induced by H_2O_2 in the presence of AA (Chapter IV). This was attributed to the loss of AA in the medium through iron-catalyzed oxidation. Moreover, AA increases H_2O_2-induced DNA damage in the presence of an excess of *apo*-transferrin, which binds iron with great affinity and keeps it in a non-reactive state (Chapter IV). Overall, the present work showed that the exacerbating effect of AA on H_2O_2-induced DNA damage does not depend on the presence of transition metal ions in the cell medium. However, the possibility that AA increased the intracellular reactive iron pool by promoting the uptake of membrane-bound iron cannot be excluded. Likewise, the possibility that the effect of AA is two-fold, *i.e.* AA may promote the uptake of extracellular NTBI and keep it in the reduced, catalytically active state inside cells, cannot be excluded.

H_2O_2 is known to affect iron homeostasis, but it is unlikely that the effects of AA on iron homeostasis reported in the present work would result from the H_2O_2 produced during the auto-oxidation of AA in the extracellular medium. First, the effects of AA on iron homeostasis were reproduced when using AA2P, which remains stable when dissolved in culture medium supplemented with 10 % FBS for up to a week (Hata *et al.* 1989). Furthermore, the results presented in Chapter III showed that AA2P did not lead to the formation of extracellular H_2O_2. Second, it has been shown that the exposure of murine fibroblasts to 100 μM H_2O_2 inhibited ferritin synthesis and increased the levels of TFRC mRNA, protein and the cell-surface expression of the receptor (Caltagirone *et al.* 2001). Goralska *et al.* (1997) have also observed that H_2O_2 markedly decreased *de novo* ferritin synthesis. So, the effects of AA on TFRC and cellular ferritin cannot be ascribed to the formation of H_2O_2 in the extracellular medium during AA auto-oxidation.

As discussed in Section 4.3.4, AA could increase oxidative DNA damage by promoting iron release from the sites of sequestration within the cell, allowing it to bind DNA, and/or by keeping DNA-bound iron in a reduced state, consequently enhancing

hydroxyl radical formation from H_2O_2. It is possible that oxidative damage formation may be gradually diminished by the induction of ferritin synthesis. In the present work, the ability of AA to enhance H_2O_2-induced cell killing was observed when cells were pre-incubated with AA for 4 hours, *i.e.* before ferritin levels were maximally increased. It is possible that the increase in cellular ferritin would provide some degree of protection at later time points. In fact, the induction of ferritin synthesis confers resistance against H_2O_2-induced cytotoxicity and may be part of an inducible protective response to oxidative stress (Balla *et al.* 1992; Lin and Girotti 1998). For example, incubation with 5 μM hemin for 1 hour sensitises endothelial cells towards H_2O_2 (100 μM). However, when cells were incubated with hemin for one hour and then further incubated in hemin-free growth medium for 15 hours, they became protected against H_2O_2-induced cytotoxicity. Hemin pre-treatment caused an approximately 10-fold increase in cellular ferritin during the same period. The authors showed that cytoprotection could be accounted for by the inducible increase in ferritin synthesis (Balla *et al.* 1992). It is unknown whether the reduction of transferrin-dependent iron uptake would confer some degree of protection.

The relevance of the pro-oxidant effect of AA *in vivo* remains to be established. Whilst it is known that biological fluids (plasma, lymph or synovial fluid) from individuals taking AA supplements have greater potential to form hydroxyl radicals after *ex vivo* addition of H_2O_2 and of an iron catalyst (Winterbourn 1981), most supplementation studies showed either an AA-mediated reduction in oxidative DNA damage or a null effect, whereas a few contentious studies showed an increase in specific base lesions (reviewed by Duarte and Lunec 2005). A study showed that AA oral supplementation significantly increased chromosome aberrations induced by *ex vivo* bleomycin challenge of lymphocytes, suggesting that AA could enhance DNA damage by modulating the iron status *in vivo* (Anderson *et al.* 1997).

The data presented in this work show that AA affects cellular iron homeostasis in HDFs and may accelerate oxidative damage caused by exogenous sources of ROS. In several inflammatory conditions, fibroblasts release chemokines, which in turn recruit leukocytes to the site of inflammation (Ritchlin 2000). The latter are capable of producing large amounts of superoxide and H_2O_2 (Halliwell and Gutteridge 1999). Likewise, iron can be released during inflammation, possibly due to the ability of superoxide to mobilise Fe^{2+}

from ferritin (Biemond *et al.* 1984). Naturally, the presence of catalytic iron can aggravate the effects of ROS species by promoting formation of more oxidising species such as hydroxyl radicals (Halliwell and Gutteridge 1999). By mobilising catalytic iron from ferritin and/or favouring the cycle of repetitive reduction of ferric iron back to ferrous iron, AA may exacerbate the formation of hydroxyl radical at sites of inflammation. In fact, a study with human volunteers showed that AA supplements augment the levels of bleomycin-detectable iron in the serum and the levels of lipid hydroperoxide during acute inflammation induced by eccentric exercise (Childs *et al.* 2001). The results obtained in the present study with HDFs further suggest that AA may accelerate the destruction of normal tissues, a commonly observed deleterious consequence of inflammatory reactions in rheumatoid arthritis and other chronic inflammatory conditions (Forman and Thomas 1986), which is thought to result from iron-mediated oxidative stress (Rowley *et al.* 1984). Tissue injury can, in turn, promote free radical reactions even further by liberating catalytic metal ions from damaged cells into the surrounding environment.

AA supplements are currently amongst the most popular vitamin and mineral supplements. Many individuals ingest grams of the vitamin to prevent heart disease, increase resistance to common cold, manage stress or simply improve well-being. In addition, it has been proposed that rheumatoid arthritis patients would benefit from antioxidant supplementation (Frei *et al.* 1988; Jaswal *et al.* 2003), since their plasma AA is oxidised at a faster rate (Blake *et al.* 1981; Jaswal *et al.* 2003). However, for the above mentioned reasons, AA supplements would likely be more detrimental than beneficial in pathological conditions associated with iron overload and inflammation by exacerbating iron-mediated tissue damage.

Gastrointestinal disturbances are the most common adverse clinical events associated with acute, high doses of vitamin C (generally above 1000 mg/day) given over a short period of time, but metabolic acidosis, oxaluria, renal stone and fatigue have also been reported (Barness 1975). Very recently, a group of scientists has re-evaluated the potential toxic risks of vitamin C supplements (Hathcock *et al.* 2005). After reviewing clinical trial data, authors claimed that for most adults vitamin C supplements are safe until 2000 mg/day. However, the authors did not consider individuals with iron overload, a group that is particularly at risk. In addition, authors only took into consideration oral

supplements and did not evaluate the risks of intravenous administration. Finally, authors admitted that there was no evidence from clinical trials that vitamin C supplements are beneficial.

7.2. Vitamin C-induced changes in gene expression

The DNA damage and cell viability experiments presented in Chapter IV showed that, in the absence of an oxidative stress, AA was harmless to HDFs. This is further supported by the fact that AA did not induce the expression of genes encoding stress response proteins or antioxidant enzymes (Chapter VI). Moreover, although non-dividing HDFs are capable of repairing DNA (Kantor and Setlow 1981), no changes in the expression of DNA repair genes were observed in the present study. Instead, results presented in Chapter VI showed that the major transcriptional response of quiescent HDFs to AA consisted of the induction of genes that are required for cell cycle re-entry and ultimately cell division. This agrees with the ability of AA to promote the proliferation of post-confluent HDFs, also observed in the current study. Results thus suggest that AA may have a growth factor-like role in non-dividing populations of HDFs. Growth factors usually bind to their receptor at the cell surface and activate specific signalling pathways. Two signalling pathways have been implicated in the proliferation of HDFs in response to mitogens, mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway (Hafizi *et al.* 1999). The PI3K pathway is activated by the binding of insulin or insulin-like growth factors to their receptors at the cell membrane (Kozma and Thomas 2002; Lizcano and Alessi 2002). Insulin has also been shown to stimulate monocyte LDLR expression *in vivo* (Duvillard *et al.* 2003) and its lipogenic action in the liver is mediated by SREBP1 (Osborne 2000; Horton *et al.* 2002). Analysis of gene profiling results in the current study revealed that a significant number of genes involved in the progression through G_2/M that were up-regulated by AA are targets for phosphoinositide-mediated signalling. Likewise, studies have implicated the PI3K signalling cascade in regulation of the G_2-M checkpoint and shown that a PI3K inhibitor down-regulates the expression of G_2-M phase genes (Le *et al.* 2005). Accordingly, inhibition of PI3K pathway abrogates mitogen-induced proliferation of primary HDFs *in vitro* (Hafizi *et al.* 1999). Future work should aim at identifying the signalling pathways

that mediate the growth-stimulatory action of AA in HDFs, the PI3K pathway being a candidate.

Iron is required for cell proliferation and for cell cycle progression from G1 to S phase (reviewed by Le and Richardson 2002). In fact, transferrin is an essential requirement for the growth of cells in culture (Morgan 1981). Iron chelation inhibits DNA synthesis, probably by inhibiting the activity of a Fe-containing protein, ribonucleotide reductase, which is the rate-limiting enzyme in the conversion of ribonucleotides into deoxyribonucleotides (Lederman *et al.* 1984). It is possible that the AA-mediated increase in intracellular LIP (described in Chapter IV) would also contribute to the ability of AA to induce cell cycle re-entry and cell division in quiescent HDFs.

As discussed in Section 6.4, AA induces a transcriptional response that is consistent with cell cycle re-entry of quiescent cells and stimulates proliferation of post-confluent, cultured HDFs. However, it remains to be determined if AA can promote fibroblast activation *in vivo*. Whilst the activation of quiescent cells back into the cell cycle has tumourigenic potential, there is no evidence that AA may induce tumour formation *in vivo*. In fact, AA was only able to induce cell-cycle re-entry of quiescent HDFS in the presence of serum. In the body, dermal fibroblasts are exposed to serum during the re-colonisation of a wound. Fibroblasts are key elements in the process of wound healing and the mitogenic activation of dermal fibroblasts may be part of their physiological response to a wound (Iyer *et al.* 1999). AA may, therefore, stimulate the process of wound healing, where quiescent dermal fibroblasts are required to rapidly undergo cell division. In fact, studies in humans and in guinea pigs have shown that vitamin C deficiency impairs wound healing and repletion of the vitamin corrects the problem (Peterkofsky 1991; Hirschmann and Raugi 1999). Although this has been attributed to the ability of AA to stimulate collagen synthesis, the current results suggest that AA may also have an important role in wound healing by stimulating fibroblast activation.

Fibroblast proliferation can also be induced *in vivo* in response to cytokines and growth factors released from inflammatory cells, potentially leading to tissue fibrosis. It would be interesting to determine whether AA supplementation could aggravate tissue fibrosis, especially as the present work proposes that AA may enhance oxidative tissue injury during inflammation.

7.3. Summary of conclusions

The present study demonstrated that AA alters cellular iron homeostasis. AA expands the intracellular catalytic iron pool of HDFs and causes a concomitant increase in cellular ferritin, as well as down-regulation of the TFRC. It can thus be concluded that, under normal conditions, AA increases iron bioavailability. This study has also shown that, under oxidative stress, AA promotes intracellular Fenton-type free radical formation reactions, enhanced iron-dependent DNA damage formation and cell killing. It is possible that AA could enhance iron mobilization from the sites of sequestration within the cell into the cytosol and nucleus, thereby enhancing oxidative damage. In addition, AA could enhance H_2O_2-induced oxidative damage by reducing DNA-bound iron, thus favouring site-specific attack on DNA. It is proposed that vitamin C may promote normal tissue injury in situations associated with elevated ROS production, such as during chronic inflammation.

These findings help elucidating the mechanisms by which the vitamin influences iron availability in human cells and suggest that the vitamin has the potential to exacerbate the deleterious effects of metals *in vivo*. Furthermore, this work presents evidence that vitamin C is able to modulate gene expression in cultured HDFs, stimulating cell cycle re-entry of quiescent cells. Although the relevance of these effects *in vivo* remains to be proved, results suggest that AA may stimulate fibroblast activation in the process of wound healing.

In summary, this work shows the effects of vitamin C on DNA damage, iron homeostasis and gene expression profiling in HDFs.

APPENDIX I

TESTING CELL LINES FOR *MYCOPLASMA* CONTAMINATION

HDFs were tested for *Mycoplasma* contamination using a PCR-based method (VenorGem Mycoplasma Detection Kit, Cambio, Cambridge, UK) as indicated in Materials and Methods. Briefly, the PCR reaction included an internal control, which consisted of plasmid DNA. In addition, each reaction included either water (no-template control), cell culture supernatant from GM5399 or GM969 HDFs, or DNA from *Mycoplasma orale* (positive control). An aliquot of the PCR product was run on a 1.5 % agarose gel, stained with EtBr and visualised in a UV transilluminator. Results are depicted in Figure 1. As expected, the internal control band was present in all samples. The band corresponding to *Mycoplasma* DNA, however, was present in the positive control and absent in the test samples. This indicates that the GM969 or GM5399 supernatants tested were free of *Mycoplasma* contamination.

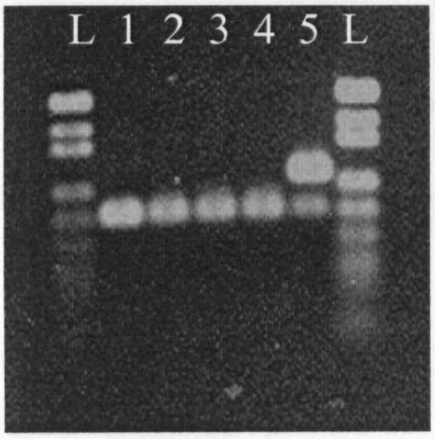


Figure 1. Testing of cell culture supernatants for *Mycoplasma* contamination. *Mycoplasma* contamination was tested using a PCR-based method, as indicated in Materials and Methods. PCR products were run on a 1.5 % agarose gel, stained with ethidium bromide and visualised in a UV transilluminator. L, DNA ladder; 1, no-template control; 2, GM969 supernatant; 3 and 4, GM5399 supernatant; 4, GM5399 supernatant; 5, positive control.

APPENDIX II

UVA LAMP SPECTRAL ANALYSIS

A custom UV box was created in order to allow uniform UV irradiation of cells growing in culture plates or dishes at an adjustable distance from the UV source and for the desired periods of time. The UV box fits a bank of six closely spaced Cleo Performance/40 watts fluorescent lamps (Philips). A Schott Desag M-UG2 UV transmitting absorption glass filter (HV Skan, Solihull, UK) was applied in order to obtain a broadband UVA spectrum free of contaminant wavelengths (UVB and visible). Furthermore, the wavelength emission spectra of these lamps (including the filtering) were characterised using a single monochromator diode array spectroradiometer, with the help of staff from the Laboratory of Atmospheric Chemistry, Department of Chemistry, University of Leicester. Spectral analysis confirmed that Cleo Performance lamps emit a broadband UVA spectrum and visible light contamination was efficiently removed by irradiating through an M-UG2 glass filter (Figure 1). Irradiation through a Petri dish lid seems to cause only a minimal shift in the UV spectrum of the lamp and mainly a reduction in irradiation intensity (Figure 2). It was thus demonstrated that a Petri dish lid could be kept during the irradiation without causing significant changes in the wavelength spectrum. This is useful in preventing microbial contamination during the irradiation procedure.

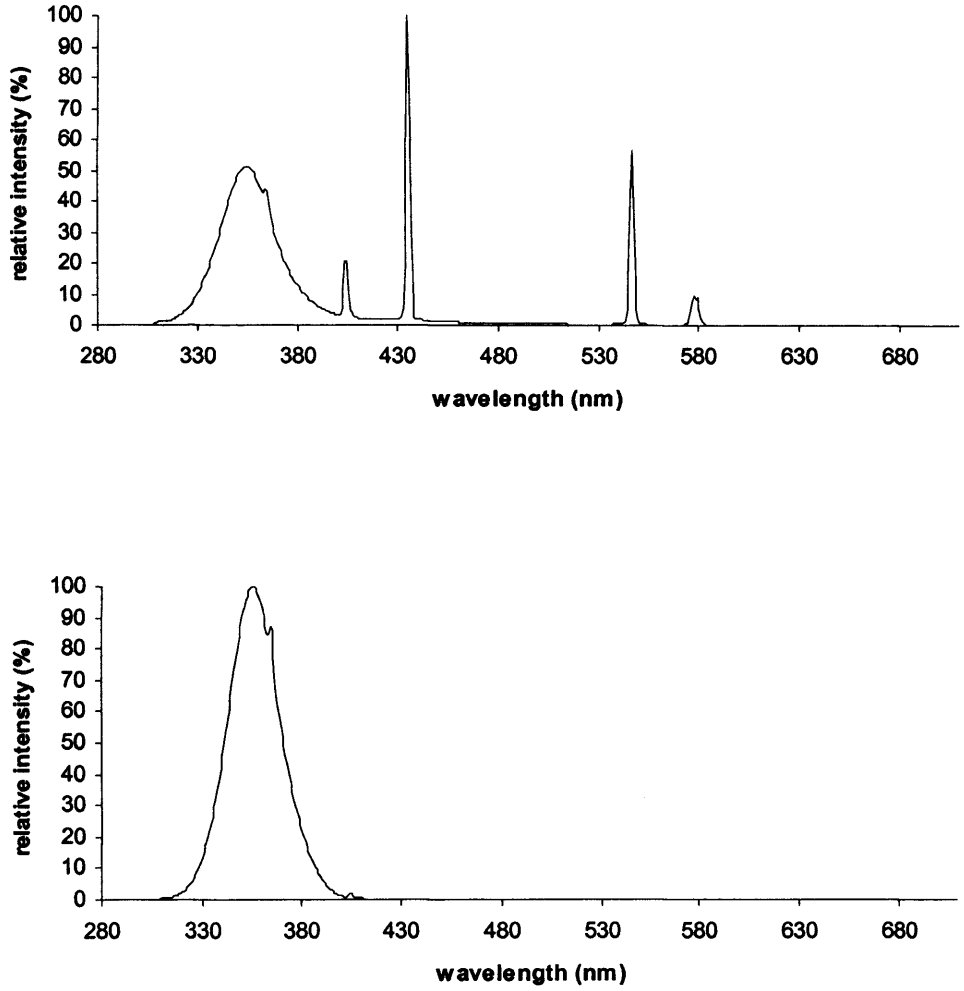


Figure 1. Spectral analysis of CLEO Performance lamps. Irradiation was performed in the absence (top) or presence (bottom) of an M-UG2 glass filter. The wavelength emission spectra were characterised using a single monochromator diode array spectroradiometer.

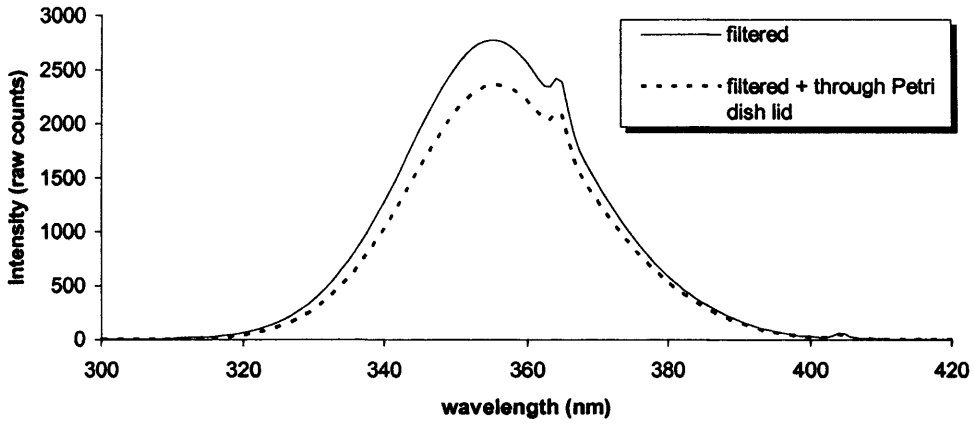


Figure 2. Spectral analysis of M-UG2-filtered CLEO Performance lamps. Irradiation was performed through an M-UG2 glass filter or through the filter and a Petri dish lid. The wavelength emission spectra were characterised using a single monochromator diode array spectroradiometer.

APPENDIX III

THE TFRC mRNA SEQUENCE

The TFRC mRNA (5 kb) includes a large (~2.5 kb) untranslated region (UTR) at the 3' end of the mRNA (Kühn et al. 1984) that is required for the iron-dependent regulation of receptor expression (Owen and Kühn 1987). The 3'-UTR includes five stem-loop structures containing IREs. The loop is a conserved sequence of six nucleotides, with a consensus sequence 5'-CAGUGN-3', situated on top of a stem composed of any paired bases. The stem has a variable length, but it invariably contains an unpaired cytidine situated six bases 5' of the six nucleotide loop (Klausner *et al.* 1993). These structures are recognised by cytosolic RNA-binding proteins, IRPs. The binding of IRPs to IREs is enhanced by iron depletion and protects the mRNA from degradation by nucleases, ultimately increasing the expression of the TFRC (reviewed by Templeton and Liu 2003; Hentze *et al.* 2004).

The TFRC mRNA sequence, including the coding domain sequence (CDS) and the 5' and 3' UTRs, is shown below. The CDS is dot-underlined. The sequences of the primers used for real-time RT-PCR amplification of the TFRC mRNA are full-underlined. IREs consensus sequence is shown in blue.

5'-GGCGGCUCGGGACGGAGGACGCGCUAGUGUGAGUGCGGGCUUCUAGAACUACACCG
ACCCUCGUGUCCUCCCUUCAUCCUGCGGGGCUGGCUGGAGCGGCCGCUCCGGUGCUGUCC
AGCAGCCAUAGGGAGCCGCACGGGGAGCGGGAAAGCGGUCGCGGCCCCAGGCGGGGCGGC
CGGGAUGGAGCGGGGCCGCGAGCCUGUGGGGAAGGGGCUGUGGCGGCGCCUCGAGCGGCU
GCAGGUUCUUCUGUGUGGCAGUUCAGAAUGAUGGAUCAAGCUAGAUCAGCAUUCUCUAA
CUUGUUUGGUGGAGAACCAUUGUCAUAUACCCGGUUCAGCCUGGCUCGGCAAGUAGAUG
GCGAUAACAGUCAUGUGGAGAUGAAACUUGCUGUAGAUGAAGAAGAAAAUGCUGACAAU
AACACAAAGGCCAAUGUCACAAAACCAAAAAGGUGUAGUGGAAGUAUCUGCUAUGGGAC
UAUUGCUGUGAUCGUCUUUUUCUUGAUUGGAUUUAUGAUUGGCUACUUGGGCUAUUGUA
AAGGGGUAGAACCAAAAACUGAGUGUGAGAGACUGGCAGGAACCGAGUCUCCAGUGAGG
GAGGAGCCAGGAGAGGACUUCCCUGCAGCACGUCGCUUAUAUUGGGAUGACCUGAAGAG
AAAGUUGUCGGAGAAACUGGACAGCACAGACUUCACCGGCACCAUCAAGCUGCUGAAUGA
AAAUUCAUAUGUCCCUCGUGAGGCUGGAUCUCAAAAAGAUGAAAAUCUUGCGUUGUAUG
UUGAAAAUCAAUUUCGUGAAUUUAAACUCAGCAAAGUCUGGCGUGAUCAACAUUUUGUU
AAGAUUCAGGUCAAAGACAGCGCUCAAAACUCGGUGAUCAUAGUUGAUAAGAACGGUAG
ACUUGUUUACCUGGUGGAGAAUCCUGGGGGUUAUGUGGCGUAUAGUAAGGCUGCAACAG
UUACUGGUAAACUGGUCCAUGCUAAUUUUGGUACUAAAAAAGAUUUUGAGGAUUUAUAC
ACUCCUGUGAAUGGAUCUAUAGUGAUUGUCAGAGCAGGGAAAAUCACCUUUGCAGAAAA
GGUUGCAAAUGCUGAAAGCUUAAAUGCAAUUGGUGUGUUGAUAUACAUGGACCAGACUA
AAUUUCCCAUUGUUAACGCAGAACUUUCAUUCUUUGGACAUGCUCAUCUGGGGACAGGU
GACCCUUACACACCUGGAUUCCCUUCCUUCAAUCACACUCAGUUUCCACCAUCUCGGUCA
UCAGGAUUGCCUAAUAUACCUGUCCAGACAAUCUCCAGAGCUGCUGCAGAAAAGCUGUUU

GGGAAUAUGGAAGGAGACUGUCCCUCUGACUGGAAAACAGACUCUACAUGUAGGAUGGU
AACCUCAGAAAGCAAGAAUGUGAAGCUCACUGUGAGCAAUGUGCUGAAAGAGAUAAAAA
UUCUUAACAUCUUUGGAGUUAUUAAAGGCUUUGUAGAACCAGAUCACUAUGUUGUAGUU
GGGGCCCAGAGAGAUGCAUGGGGCCCUGGAGCUGCAAAAUCCGGUGUAGGCACAGCUCUC
CUAUUGAAACUUGCCCAGAUGUUCUCAGAUAUGGUCUUAAAAGAUGGGUUUCAGCCCAG
CAGAAGCAUUAUCUUUGCCAGUUGGAGUGCUGGAGACUUUGGAUCGGUUGGUGCCACUG
AAUGGCUAGAGGGAUACCUUUCGUCCCUGCAUUUAAAGGCUUUCACUUAUAUUAAUCUG
GAUAAAGCGGUUCUUGGUACCAGCAACUUCAAGGUUUCUGCCAGCCCACUGUUGUAUACG
CUUAUUGAGAAAACAAUGCAAAAUGUGAAGCAUCCGGUUACUGGGCAAUUUCUAUAUCA
GGACAGCAACUGGGCCAGCAAAGUUGAGAAACUCACUUUAGACAAUGCUGCUUUCCCUUU
CCUUGCAUAUUCUGGAAUCCCAGCAGUUUCUUUCUGUUUUUGCGAGGACACAGAUUAUCC
UUAUUUGGGUACCACCAUGGACACCUAUAAGGAACUGAUUGAGAGGAUUCCUGAGUUGA
ACAAAGUGGCACGAGCAGCUGCAGAGGUCGCUGGUCAGUUCGUGAUUAAACUAACCCAU
GAUGUUGAAUUGAACCUGGACUAUGAGAGGUACAACAGCCAACUGCUUUCAUUUGUGAG
GGAUCUGAACCAAUACAGAGCAGACAUAAAGGAAAUGGGCCUGAGUUUACAGUGGCUGU
AUUCUGCUCGUGGAGACUUCUUCCGUGCUACUUCCAGACUAACAACAGAUUUCGGGAAUG
CUGAGAAAACAGACAGAUUUGUCAUGAAGAAACUCAAUGAUCGUGUCAUGAGAGUGGAG
UAUCACUUCCUCUCUCCCUACGUAUCUCCAAAAGAGUCUCCUUUCCGACAUGUCUUCUGG
GGCUCCGGCUCUCACACGCUGCCAGCUUUACUGGAGAACUUGAAACUGCGUAAACAAAAU
AACGGUGCUUUUAAUGAAACGCUGUUCAGAAACCAGUUGGCUCUAGCUACUUGGACUAU
UCAGGGAGCUGCAAAUGCCCUCUCUGGUGACGUUUGGGACAUUGACAAUGAGUUUUAAA
UGUGAUACCCAUAGCUUCCAUGAGAACAGCAGGGUAGUCUGGUUUCUAGACUUGUGCUG
AUCGUGCUAAAUUUUCAGUAGGGCUACAAAACCUGAUGUUAAAAUUCCAUCCCAUCAUC
UUGGUACUACUAGAUGUCUUUAGGCAGCAGCUUUUAAUACAGGGUAGAUAACCUGUACU
UCAAGUUAAAGUGAAUAACCACUUAAAAAAUGUCCAUGAUGGAAUAUUCCCCUAUCUCU
AGAAUUUUAAGUGCUUUGUAAUGGGAACUGCCUCUUUCCUGUUGUUGUUAAUGAAAAUG
UCAGAAACCAGUUAUGUGAAUGAUCUCUCUGAAUCCUAAGGGCUGGUCUCUGCUGAAGG
UUGUAAGUGGUCGCUUACUUUGAGUGAUCCUCCAACUUCAUUUGAUGCUAAAUAGGAGA
UACCAGGUUGAAAGACCUUCUCCAAAUGAGAUCUAAGCCUUUCCAUAAGGAAUGUAGCU
GGUUUCCUCAUUCCUGAAAGAAACAGUUAACUUUCAGAAGAGAUGGGCUUGUUUUCUUG
CCAAUGAGGUCUGAAAUGGAGGUCCUUCUGCUGGAUAAAAUGAGGUUCAACUGUUGAUU
GCAGGAAUAAGGCCUUAAUAUGUUAACCUCAGUGUCAUUUAUGAAAAGAGGGGACCAGA
AGCCAAAGACUUAGUAUAUUUUCUUUUCCUCUGUCCCUUCCCCCAUAAGCCUCCAUUUAG
UUCUUUGUUAUUUUUGUUUCUUCCAAAGCACAUUGAAAGAGAACCAGUUUCAGGUGUUU
AGUUGCAGACUCAGUUUGUCAGACUUUAAAGAAUAAUAUGCUGCCAAAUUUUGGCCAAA
GUGUUAAUCUUAGGGGAGAGCUUUCUGUCCUUUUGGCACUGAGAUAUUUAUUGUUUAUU
UAUCAGUGACAGAGUUCACUAUAAAUGGUGUUUUUUUAAUAGAAUAUAAUUAUCGGAA
GCAGUGCCUUCCAUAAUUAUGACAGUUAUACUGUCGGUUUUUUUUAAAUAAAAGCAGCA
UCUGCUAAUAAAACCCAACAGAUACUGGAAGUUUUGCAUUUAUGGUCAACACUUAAGGG
UUUUAGAAAACAGCCGUCAGCCAAAUGUAAUUGAAUAAAGUUGAAGCUAAGAUUUAGAG

AUGAAUUAAAUUUAAUUAGGGGUUGCUAAGAAGCGAGCACUGACCAGAUAAGAAUGCUG
GUUUUCCUAAAUGCAGUGAAUUGUGACCAAGUUAUAAAUCAAUGUCACUUAAAGGCUGU
GGUAGUACUCCUGCAAAAUUUUAUAGCUCAGUUUAUCCAAGGUGUAACUCUAAUUCCCA
UUUUGCAAAAUUUCCAGUACCUUUGUCACAAUCCUAACACAUUAUCGGGAGCAGUGUCU
UCCAUAAUGUAUAAAGAACAAGGUAGUUUUUACCUACCACAGUGUCUGUAUCGGAGACA
GUGAUCUCCAUAUGUUACACUAAGGGUGUAAGUAAUUAUCGGGAACAGUGUUUCCCAUA
AUUUUCUUCAUGCAAUGACAUCUUCAAAGCUUGAAGAUCGUUAGUAUCUAACAUGUAUC
CCAACUCCUAUAAUUCCCUAUCUUUUAGUUUUAGUUGCAGAAACAUUUUGUGGUCAUUA
AGCAUUGGGUGGGUAAAUUCAACCACUGUAAAAUGAAAUUACUACAAAAUUUGAAAUUU
AGCUUGGGUUUUUGUUACCUUUAUGGUUUCUCCAGGUCCUCUACUUAAUGAGAUAGUAG
CAUACAUUUAUAAUGUUUGCUAUUGACAAGUCAUUUUAACUUUAUCACAUUAUUUGCAU
GUUACCUCCUAUAAACUUAGUGCGGACAAGUUUUAAUCCAGAAUUGACCUUUUGACUUA
AAGCAGAGGGACUUUGUAUAGAAGGUUUGGGGGCUGUGGGGAAGGAGAGUCCCCUGAAG
GUCUGACACGUCUGCCUACCCAUUCGUGGUGAUCAAUUAAAUGUAGGUAUGAAUAAGUU
CGAAGCUCCGUGAGUGAACCAUCAUUAUAAACGUGAUGAUCAGCUGUUUGUCAUAGGGC
AGUUGGAAACGGCCUCCUAGGGAAAAGUUCAUAGGGUCUCUUCAGGUUCUUAGUGUCAC
UUACCUAGAUUUACAGCCUCACUUGAAUGUGUCACUACUCACAGUCUCUUUAAUCUUCAG
UUUUAUCUUUAAUCUCCUCUUUUAUCUUGGACUGACAUUUAGCGUAGCUAAGUGAAAAG
GUCAUAGCUGAGAUUCCUGGUUCGGGUGUUACGCACACGUACUUAAAUGAAAGCAUGUG
GCAUGUUCAUCGUAUAACACAAUAUGAAUACAGGGCAUGCAUUUUGCAGCAGUGAGUCU
CUUCAGAAAACCCUUUUCUACAGUUAGGGUUGAGUUACUUCCUAUCAAGCCAGUACGUGC
UAACAGGCUCAAUAUUCCUGAAUGAAAUAUCAGACUAGUGACAAGCUCCUGGUCUUGAG
AUGUCUUCUCGUUAAGGAGAUGGGCCUUUUGGAGGUAAAGGUAUA-3'

APPENDIX IV

PUBLICATIONS AND COMMUNICATIONS IN SCIENTIFIC MEETINGS

A – Thesis-related work:

i) Publications:

Duarte, T. L. & Jones, G. D. D. (Submitted) Vitamin C modulation of H_2O_2-induced damage and iron homeostasis in human cells.

Duarte, T. L., Almeida, G. M. & Jones, G. D. D. (Submitted) Investigation of the role of extracellular H_2O_2 and metal ions in the pro-oxidant effect of ascorbic acid in cell culture models.

Duarte, T. L. & Jones, G. D. D. (In preparation) Effects of ascorbic acid on gene expression profiling in human fibroblasts.

Duarte, T. L. & Lunec, J. 2005. When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. *Free Radical Research,* **39**, pp. 671-686.

ii) Communications in scientific meetings:

Duarte, T. L., Jones, G. D. D. & Lunec, J. "Vitamin C modulation of H_2O_2-induced DNA damage in human fibroblasts: a consequence of altered iron metabolism?" (Poster). "Annual meeting of the Society for Free Radical Research- Europe, Free radicals and disease processes", 8-11 July 2005. De Vere Belfry Hotel, West Midlands, UK.

Duarte, T. L., Lowes, D. A., Butler, J., Halligan, E. & Lunec, J. "Microarray analysis of vitamin C induced gene expression in human fibroblasts." (Poster). "6th Annual European Affymetrix User Group Meeting", 27-28 May 2003. Lisbon, Portugal.

Duarte, T. L., Lowes D. A., Butler J. & Lunec J. "Microarray analysis of *in vitro* vitamin C induced gene expression of human fibroblasts." (Oral Communication). "European

Research on Functional Effects of Dietary Antioxidants: Benefits and Risks", 25-28 September 2002. Cambridge, UK.

B - Collaborative work:

i) Publications:

Cooke, M. S., Singh, R., Hall, G. K., Mistry, V., **Duarte, T. L.**, Farmer, P. B. & Evans, M. D. 2006. Evaluation of ELISA and LC-MS/MS methodology for the analysis of 8-oxo-7,8-dihydro-2'-deoxyguanosine in saliva and urine. *Free Radical Biology and Medicine*, **41**, pp.1829-1836.

Almeida, G. M., **Duarte, T. L.**, Steward, W. P. & Jones, G. D. D. 2006. Detection of oxaliplatin-induced DNA crosslinks *in vitro* and in cancer patients using the Alkaline Comet Assay. *DNA Repair*, **5**, pp. 219-225.

ii) Communications in scientific meetings:

Almeida, G. M., **Duarte, T. L.**, Farmer, P. B., Steward, W. P. & Jones G. D. D. "Multiple end-point analysis reveals cisplatin damage tolerance to be a chemoresistant mechanism in a NSCLC model" (Meeting abstract). "The British Toxicology Society & The United Kingdom Environmental Mutagen Society Joint Congress", 19-22 March 2006. University of Warwick, UK.

Dove, R., **Duarte, T.**, Halligan, E., Mistry, N. & Lunec, J. "2-Ascorbate phosphate induces gene expression without pro-oxidation" (Poster). "Annual meeting of the Society for Free Radical Research- Europe, Free radicals and disease processes", 8-11 July 2005. De Vere Belfry Hotel, West Midlands, UK.

Lunec, J., **Duarte, T.**, Holloway, K. A., Griffiths, H. R. and Faux, S. P. (2004) Ascorbic acid induces an oxidative stress in CCRF cells activating the transcription factors AP-1 and NFkB (Meeting Abstract). Free Radical Biology and Medicine, 36: S29-S29.

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