OXIDATION OF ASCORBATE BY PROTEIN RADICALS IN SIMPLE SYSTEMS AND IN CELLS

Chia-chi Liu
MSc (Biotechnology) UNSW, Australia

Department of Chemistry and Biomolecular Sciences
Macquarie University
Sydney, NSW 2109

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SUMMARY

Generation of peroxide groups in proteins exposed to a wide variety of reactive oxygen species (ROS) requires an initial formation of protein carbon-centred or peroxyl free radicals, which can be reduced to hydroperoxides. Both protein radicals and protein hydroperoxides are capable of oxidizing important biomolecules and thus initiate biological damage. In this study, we investigated the inhibition of protein hydroperoxide formation by ascorbate and GSH in gamma-irradiated HL-60 cells.

We used HL-60 cells as a model for general protection of living organisms by ascorbate (Asc) and glutathione (GSH) from the deleterious effects of protein hydroperoxides generated by radicals produced by gamma radiation. Measurement by HPLC indicated that incubation of HL-60 cells with Asc in the presence of ascorbate oxidase resulted in the accumulation of intracellular Asc. The intracellular Asc levels were lowered by irradiation, demonstrating intracellular consumption of Asc by the radiation-generated radicals. Exposure of HL-60 cells to increasing gamma irradiation doses resulted in increasing accumulation of protein peroxides in the cells. This was measured by the FOX assay. A significant decrease in intracellular protein hydroperoxides was noted when the cells were treated with ascorbic acid before irradiation. A dose-dependent protective effect of Asc was observed. Asc loading also provided strong protection from radiation-generated protein hydroperoxides independently of the composition of the external medium, showing that only the radicals formed within the cells were effective in oxidizing the cell proteins. Similarly, protein peroxidation was inhibited in cells with

enhanced levels of GSH and increased when the intracellular GSH concentration was reduced. These findings indicate that ascorbate and GSH are important antioxidants in protecting cells from oxidative stress associated with the generation of protein hydroperoxide.

DECLARTION

This thesis contains no material which has been presented or accepted for

the award of any degree or diploma in any other university or institution.

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Chia-chi Liu

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ABBREVIATIONS

AAPH   2, 2'-azobis-(2-amidinopropane) dihydrochloride

ABTS   2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]

AD   Alzheimer's disease

ALS   amyotrophic lateral sclerosis

AP-1   activator protein-1

Asc   ascorbate

BSA   bovine serum albumin

BSO   L-buthionine sulfoximine

Cg   cytosine glycol

CuZnSOD   copper/zinc superoxide dismutase

CYPs   cytochrome P-450 enzyme

DHA   dehydroascorbic acid

DMPO   5, 5-dimethyl-1-pyroline-1-oxide

DMSO   dimethylsulfoxide

DTNB   5-5'-dithiobis-(2-nitrobenzoic acid)

DTT   dithiothreitol

EC-SOD   extracellular superoxide dismutase

EDTA   ethylenediaminetetracetic acid

EPR   electron paramagnetic resonance spectroscopy

ESR   electron spin resonance

FCS   fetal calf serum

FMN	flavin mononucleotide
5-FoUra	5-formyluracil
GCS	γ-glutamylcysteine synthetase
GLUT	glucose transporters
GPx	glutathione peroxidases
GRD	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GSTs	glutathione S-transferases
HNE	4-hydroxy-2-nonenal
5-HO-Cyt	5-hydroycytosine
8-HO-dG	8-hydroxy-2'-deoxyguanosine
5-HOMeUra	5-hydroxylmetyluracil
5-HO-Ura	5-hydroyuracil
HPLC	high performance liquid chromatography
HSA	human serum albumin
JUK	c-Jun N-terminal kinase
LDL	low-denisty lipoprotein
Lyz, LZ	lysozyme
Mb	myoglobin
MCO	metal-catalyzed oxidation
MDA	malondialdehyde
Mn-SOD	manganese superoxide dismutase

MPO	myeloperoxidase
MTT	3-(4,5)-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
NADPH	nicotinamide-adenine dinucleotide phosphate
NF-κB	nuclear factor B
8-oxo-dG	8-oxo-2'-deoguanosine
PBN	α-phenyl-N-tert-butyl nitrone
PCA	Perchloric acid
PD	Parkinson disease
PDH	pyruvate dehydrogenase kinase
6-PGD	6-phosphogluconate dehydrogenase
PKA	protein kinase A
PUFA	polyunsaturated fatty acid
Ptx	pentoxifylline
RA	rheumatoid arthritis
ROS	reactive oxygen species
SCEs	sister chromatid exchanges
SLE	systemic lupus erythematosis
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxy
TEMPOL	4-hydroxy-2 2,6,6-tetramethylpiperidine-1-oxy

Tg	thyroglobulin
UQ$^{\bullet-}$	ubisemiquinone anion radical
UV	ultraviolet
XO	xylenol orange

CONFERENCES & PUBLICTAIONS

The following contains a list of abstracts from scientific meetings and the publication from this study.

Conferences

Gebicki, J. M., Domazou, A., Nauser, T., Tweeddale, H, Liu, C. C., and Koppenol, W. H. Interaction of protein radicals with GSH and ascorbate in vitro and in cells. The 15th Annual Conference of the Society for Free Radical Research (Australasia), Perth, Australia, Dec 2006.

Gebicki, J. M., Domazou, A., Nauser, T., Tweeddale, H, Liu, C. C., and Koppenol, W. H. Oxidation of glutathione and ascorbate by protein radicals. 41st meeting of the Polish Biochemical Society, Bialystok, Poland, Sep 2006.

Liu, C. C. and Gebicki, J. M. Inhibition of protein peroxidation by ascorbate in gamma-irradiated HL-60 cells. The 13th Biennial Congress: International Society for Free Radical Research, Davos, Switzerland, Aug 2006.

Liu, C. C. and Gebicki, J. M. Oxidation of ascorbate by protein radicals. The 13th Annual Conference of the Society for Free Radical Research (Australasia), Christchurch, New Zealand, Dec 2005.

Publications

Gebicki, J. M., Domazou, A., Nauser, T., Tweeddale, H, Liu, C. C., and Koppenol, W. H. Oxidation of glutathione and ascorbate by protein radicals. Manuscript in preparation.

Liu, C. C. and Gebicki, J. M. The role of ascorbate and glutathione in protein peroxide formation in HL-60 cells. Manuscript in preparation.

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CHAPTER 1

<u>INTRODUCTION</u>

Free radicals are normally very reactive chemical entities possessing an unpaired electron. They are produced continuously in cells mostly as by-products of intracellular metabolism, or by leakage from mitochondrial respiration. The most common free radicals in aerobic cells belong to the class of reactive oxygen species (ROS). Reactive oxygen species are formed and degraded by all aerobic organisms which require molecular oxygen as an electron acceptor for energy production. Physiological concentration of ROS is required for normal cell function, but the state of excessive quantities of ROS is called oxidative stress. Intracellular production of these oxygen radical intermediates threatens the integrity of various biomolecules including proteins (Davies 1987), lipids (Muralikrishna Adibhatla and Hatcher 2006), and DNA (Evans, Dizdaroglu et al. 2004). This may lead to cell death and tissue injury (Davies 1995).

To combat attack from ROS and other free radicals, living cells have acquired a number of defenses. This defense takes the form of low molecular weight compounds, antioxidants such as vitamin C, vitamin E and GSH, which intercept free radicals, becoming radicals themselves, although less reactive, preventing damage to cellular biomolecules. Also, more complex approaches involve enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, which have evolved to limit the levels of ROS (Evans, Dizdaroglu et al. 2004).

Of all the components of the cell, proteins are the mostly likely primary targets of ROS, not DNA, lipid and carbohydrates (Gieseg 2000). This study has focused on ROS–induced damage to proteins and the influences of protein oxidative intermediates on small molecular antioxidants, such as ascorbic acid. The inert natures of most of the products of protein oxidation has determined research priorities on their identification and removal, but the recent study of protein peroxides and their radical derivatives imply that protein oxidation may play a essential role in diseases induced by free radicals and other reactive oxygen species (Du 2002).

1.1 Reactive oxygen species (ROS)

1.1.1 What are ROS?

Reactive oxygen species include a class of free radicals with unpaired electrons residing predominantly on an oxygen atom (Maritinez-Cayuela 1995; Dreher 1996; Halliwell 1999; Fang 2002; Pratico 2002). Some of the ROS are extremely reactive, such as the hydroxyl radical, while some are less reactive (superoxide and hydrogen peroxide) (Pryor 1986; Korsmeyer 1995). ROS can be classified into two groups of compounds, radicals and nonradicals. The radical group includes compounds such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($HO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$) as shown in Table 1 (Pryor 1986; Halliwell and Gutteridge 1999; Simon 2000; Schnackenberg 2002). The group of nonradical compounds contains large variety of substances, some of which are also quite reactive (Table 1.1).

Table 1.1 Reactive oxygen species

Radicals	Non-Radicals
Superoxide anion radical, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
Hydroxyl radical, $HO^{\bullet}$	Singlet oxygen, $^1O_2$
Alkoxyl radical, $RO^{\bullet}$	Ozone, O_3
Peroxyl radical, $ROO^{\bullet}$	Hypochlorous acid, HOCl
Hydroperoxyl radical, $HOO^{\bullet}$	Peroxynitrite, $ONOO^-$
	Lipid hydroperoxide, LOOH
	Protein hydroperoxide, PrOOH

(Modified from Halliwell, B. and Gutteridge, J. M. C. 1999)

Most radicals are short-lived species with typically a very short half-life (10^{-2}~10^{-9} second) and low steady-state concentration, because they are extremely unstable and reactive. For instance, $HO^{\bullet}$ can survive for 10^{-9} sec in biological systems with extremely high reaction rate constant (10^7-10^{11} $m^{-1}s^{-1}$) for biological compounds. $RO^{\bullet}$ can survive about 10^{-6} of a second, while the half-life of $ROO^{\bullet}$ is about 7 seconds (Pryor 1986; Kohen and Nyska 2002). $HO^{\bullet}$, a highly reactive species, is produced in locations where it can cause damage by interacting with its immediate surroundings. However, the relatively long half-life of superoxide radicals allows them to move to locations where they can undergo interaction with other molecules (Kohen and Nyska 2002).

The species investigated in this study are superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($HO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), hydroperoxyl radicals ($HOO^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$) and protein hydroperoxides.

1.1.2 The major reactive oxygen species

The major ROS molecules and their metabolic fate are listed in Table 1.2 (Nordberg and Arner 2001).

Superoxide anion radicals ($O_2^{\bullet-}$)

The superoxide anion generated from molecular oxygen by the addition of an electron is not highly reactive for a free radical (reaction 1.1).

$$O_2 + e_{aq}^- \rightarrow O_2^{\bullet-} \tag{1.1}$$

About 1-2% of oxygen consumption results in the production of superoxide anion in aerobic metabolism. It lacks of the ability to penetrate lipid membranes and is therefore enclosed in the compartment where it was produced. The formation of superoxide takes place spontaneously, especially in the electron rich-aerobic environment in vicinity of the inner mitochondrial membrane with respiratory chain. Superoxide is also produced endogenously by enzymes, such as xanthine oxidase (Nordberg and Arner 2001). It follows that superoxide radicals are the earliest formed and the most abundant radicals in cells. Superoxide anion can interact with other molecules to generate other ROS.

The major type of reaction of this radical is reduction, for instance, Fe^{3+} and Cu^{2+} reduction (Reaction 1.2 and 1.3). Thus, this reactive species is able to induce other reactive chemicals generation, such as when it reacts spontaneously with itself or via enzyme-catalysis yielding hydrogen peroxide (H_2O_2) (Pryor 1986; Maritinez-Cayuela 1995; Dreher 1996).

$$O_2^{\bullet-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \tag{1.2}$$

$$O_2^{\bullet-} + Cu^{2+} \rightarrow O_2 + Cu^+ \tag{1.3}$$

$$2\ O_2{}^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + O_2 \qquad\qquad (1.4)$$
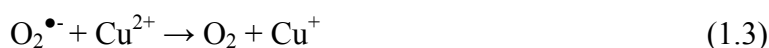
Table 1.2 The major ROS molecules and their metabolism

ROS molecule	Main sources	Enzymatic defense systems	Product(s)
Superoxide anion $(O_2{}^{\bullet-})$	'Leakage' of electrons from the electron transport chain Activated phagocytes Xanthine oxidase Flavoenzymes	Superoxide dismutase (SOD)	$H_2O_2 + O_2$
Hydrogen peroxide (H_2O_2)	From $O_2{}^{\bullet-}$ via superoxide dismutase (SOD) NADPH-oxidase (neutrophils) Glucose oxidase	Glutathione peroxidase Catalases Peroxiredoxins (Prx)	$H_2O + GSSG$ $H_2O + O_2$ $H_2O + O_2$
Hydroxyl radical $(HO^{\bullet})$	From $O_2{}^{\bullet-}$ and H_2O_2 via transition metals (Fe or Cu)	None	

(Table is from Nordberg, J. and Arner, E. S. (2001). "Reactive oxygen species, antioxidants, and the mammalian thioredoxin system." <u>Free Radic Biol Med</u> **31**(11): page 1288).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide, not a radical, is relatively stable and able to move across biological membranes and cause damage to the cell at a relatively low concentration (µM) (Maritinez-Cayuela 1995; Fleschin 2000). It plays a radical forming role as an intermediate in the production of more reactive ROS molecules including HOCl (hypochlorous acid) by the action of myeloperoxidase (Winterbourn, Vissers et al. 2000), an enzyme present in the phagosomes of neutrophils and, most importantly, formation of hydroxyl radicals ($HO^{\bullet}$) in

Fenton reaction via oxidation of transition metals. Furthermore, H_2O_2 has an important function as an intracellular signalling molecule (Rhee 1999). Direct activities of H_2O_2 include degradation of heme proteins, release of iron, inactivation of enzymes and oxidation of DNA, lipids, -SH groups and keto acids (Kohen and Nyska 2002). H_2O_2 is removed by at least three antioxidant enzyme systems, namely, catalase, glutathione peroxidases, and peroxiredoxin (Mates, Perez-Gomez et al. 1999; Sorg 2004).

Hydroxyl radicals (HO•)

The hydroxyl radical (HO•) is an extremely reactive and most oxidative species, which can rapidly react with any biological molecule (rate constants 10^7-10^{11} $M^{-1}s^{-1}$) and form other radicals (especially peroxyl radicals) in cells. It has a half-life in aqueous solution of less than 1ns, thus when produced in vivo it reacts close to its site of formation (Valko, Rhodes et al. 2006). Due to its high reactivity with biomolecules, HO• is probably capable of doing more damage to biological systems than any other ROS (Betteridge 2000). It can be generated in a multitude of different reactions, such as the interaction of high energy radiation with water, UV photolysis of hydrogen peroxide or as a byproduct of one-electron reduction of hydrogen peroxide. In addition, there are two metal-catalyzed reactions which can produce hydroxyl radical, namely the Haber-Weiss reaction (reaction 1.5) and the Fenton reaction (reaction 1.7) (Maritinez-Cayuela 1995; Dreher 1996).

$$O_2^{•-} + H_2O_2 \rightarrow O_2 + OH^- + HO^• \qquad (1.5)$$

$$Fe^{3+} / Cu^{2+} + O_2^{•-} \rightarrow Fe^{2+} / Cu^+ + O_2 \qquad (1.6)$$

$$Fe^{2+} / Cu^+ + H_2O_2 \rightarrow Fe^{3+} / Cu^{2+} + OH^- + HO^• \qquad (1.7)$$
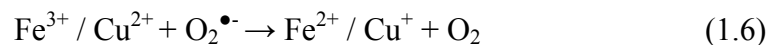
In biological system, the catalytic metal ions are usually bound in a complex with proteins or other molecules. Superoxide plays an important role in HO$^\bullet$ formation by recycling the metal ions and forming H_2O_2. Thus, metal ions play an important role in the formation of hydroxyl radicals (Halliwell 1987; Halliwell 1999). Transition metal ions can be released from proteins, such as ferritin (Harris, Cake et al. 1994).

1.2 The generation of reactive oxygen species *in vivo*

Living organisms continuously produce reactive oxygen species (ROS) (Maritinez-Cayuela 1995). ROS are generated from both endogenous and endogenous sources (Fig. 1.1). They are produced by a wide range of intracellular pathways including leakage of electron from electron transport chains (mitochondrial and endoplasmic reticulum) (Kukielka 1995; Raha 2000; Raha 2002), in a number of enzymatic reactions (e.g. lipoxygenase, cyclooxygenase and peroxidase) (Maritinez-Cayuela 1995), as a secretion of activated leukocytes (Hessel 2000), as by-products from metabolism and autooxidation of xenobiotics and as the product of stresses such as excess exercise, trauma, ischemia/reperfusion, atherosclerosis or infection. In addition, extracellular agents such as heat, freezing, radiation, chemical toxics and ultrasound generate ROS (Gebicki 2000). The activities of a number of key antioxidant enzymes, such as catalase, superoxide dismutase and glutathione peroxidase, modulate the ROS production and protect against their damaging effects (Linton, Davies et al. 2001) (Fig. 1.1).
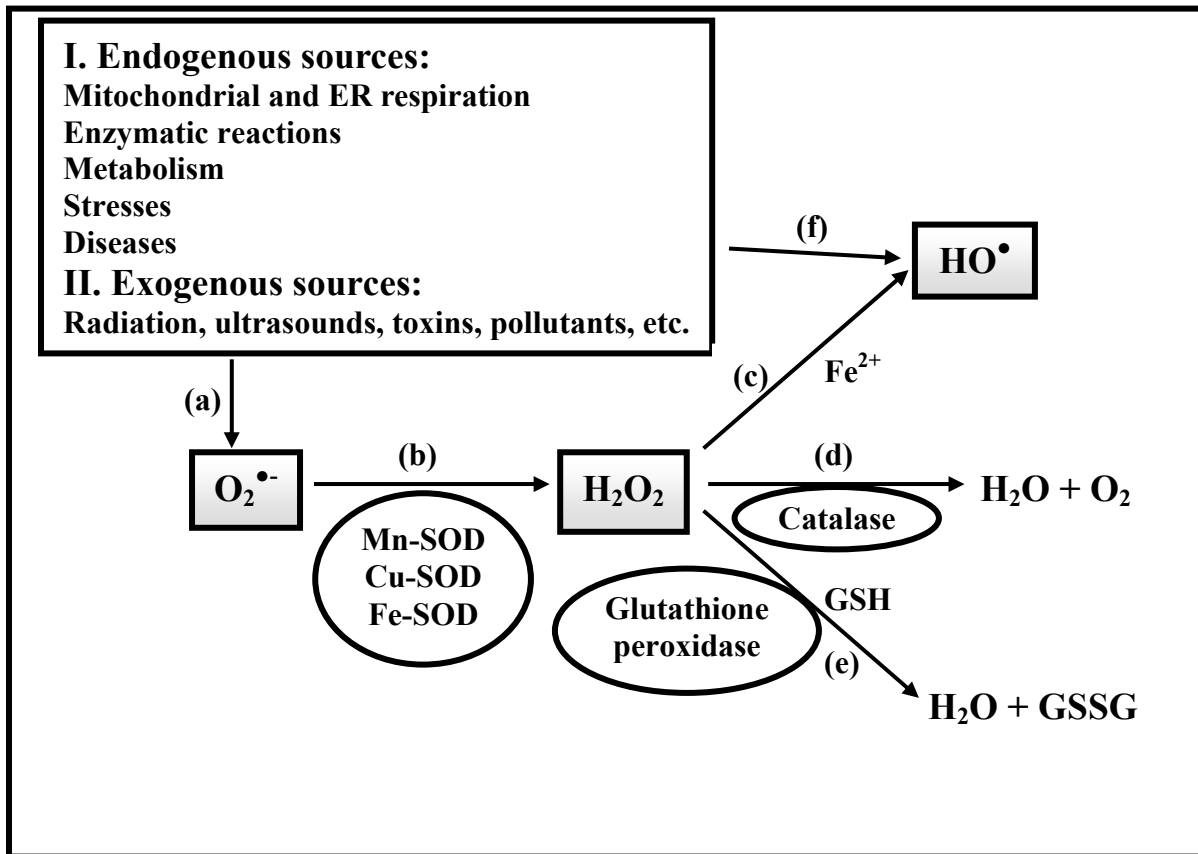
Figure 1.1 Production and degradation of oxygen free radicals

(a) The superoxide anion ($O_2^{\bullet-}$) can be produced by a variety of cellular processes. (b) The spontaneous gain of an electron to form hydrogen peroxide (H_2O_2) can be catalyzed by three isoforms of superoxide dismutase (SOD) containing manganese, copper and iron. (c) In the presence of catalytic iron, hydroxyl radical ($OH^{\bullet}$) is formed from H_2O_2 via the Fenton reaction. H_2O_2 can be decomposed to water by (d) catalase or (e) glutathione peroxidase. (f) Hydroxyl radicals are also formed by environmental factors. The common oxygen free radicals are shown in boxes and the enzyme catalysts are shown in ovals. GSH, reduced glutathione and GSSG, oxidized glutathione.

(The figure is modified from: Schnackenberg, C. G. (2002) Oxygen radicals in cardiovascular–renal disease. *Current Opinion in Pharmacology* 2, page: 122.)

1.2.1 Endogenous sources

Exposure of the organism to ROS from endogenous sources is very important and intensive; as it is a continuous process during the life span of every cell in the organism.

1.2.1.1 Mitochondrial Electron Transport System

Approximately 90% of the vast majority of cellular ROS are produced in the mitochondria (Cadenas and Davies 2000; Gille and Nohl 2001; Balaban, Nemoto et al. 2005). The electron transport chain present in the inner mitochondrial membrane achieves the energy production (ATP) via a stepwise oxidative phosphorylation, which is carried out by four electron-transporting complexes (I-IV) and one H^+-translocating ATP synthetic complex (complex V). This process uses the controlled oxidation of NADH or $FADH_2$ to generate a potential energy for protons ($\Delta\Psi$) translocated across the mitochondrial inner membrane. This potential energy is in turn used to phosphorylate ADP via the F1-F0 ATPase. Electrons derived from NADH or FADH can directly react with oxygen and generate free radicals (Newmeyer and Ferguson-Miller 2003). Mitochondrial electron transport generates superoxide ($O_2^{\bullet-}$) as an inevitable by-product and primary ROS at two complexes, Complex I (NADH CoQ oxidoreductase) and Complex III (the ubiquinol-cytochrome *c* oxidoreductase) (Dean 1987; Hagen 1989; Ames 1995; Raha 2000).

Complex I as a source of superoxide

In 2002, Raha and his colleagues reported that the major site for superoxide anion ($O_2^{\bullet-}$) production is modest leakage from mitochondrial Complex I. The rate of electron leakage from mitochondrial Complex I strongly depends on the oxygen concentration. Complex I is

a multisubunit complex which is composed of ~ 46 proteins with a combined molecular weight exceeding 1MDa and contains one non-covalently bound flavin mononucleotide (FMN) and eight iron-sulfur groups. It is also the first complex in the mitochondrial respiratory enzymatic chain. Both FMN sites and the iron-sulfur groups have been implicated in ROS generation (Newmeyer and Ferguson-Miller 2003; Jezek and Hlavata 2005) (Fig. 1.2). $O_2^{\bullet-}$ radicals release within Complex I to the matrix side has been previously observed in the experiments with submitochondrial particles and is undoubtedly compatible with the matrix location of manganese superoxide dismutase (Mn-SOD), the enzyme essential for life. This enzyme is also a regulatory control point, which allows reducing equivalents to enter the electron transport chain (Kowaltowski and Vercesi 1999).

In addition, the electron flow through Complex I and the rate of ROS production is regulated by phosphorylation events which are modulated by both protein kinase A (PKA) and pyruvate dehydrogenase kinase (PDH) in mitochondria. Complex I, which is phosphorylated by cAMP dependent PKA, can be fully active resulting in a decrease in superoxide production. Complex I phosphorylation with PDH kinase leads to a decreased enzyme activity and an increase in superoxide production. Consequently, the ROS production is probably mediated by the activation or inhibition of the complex (Cadenas and Davies 2000; Raha 2002).
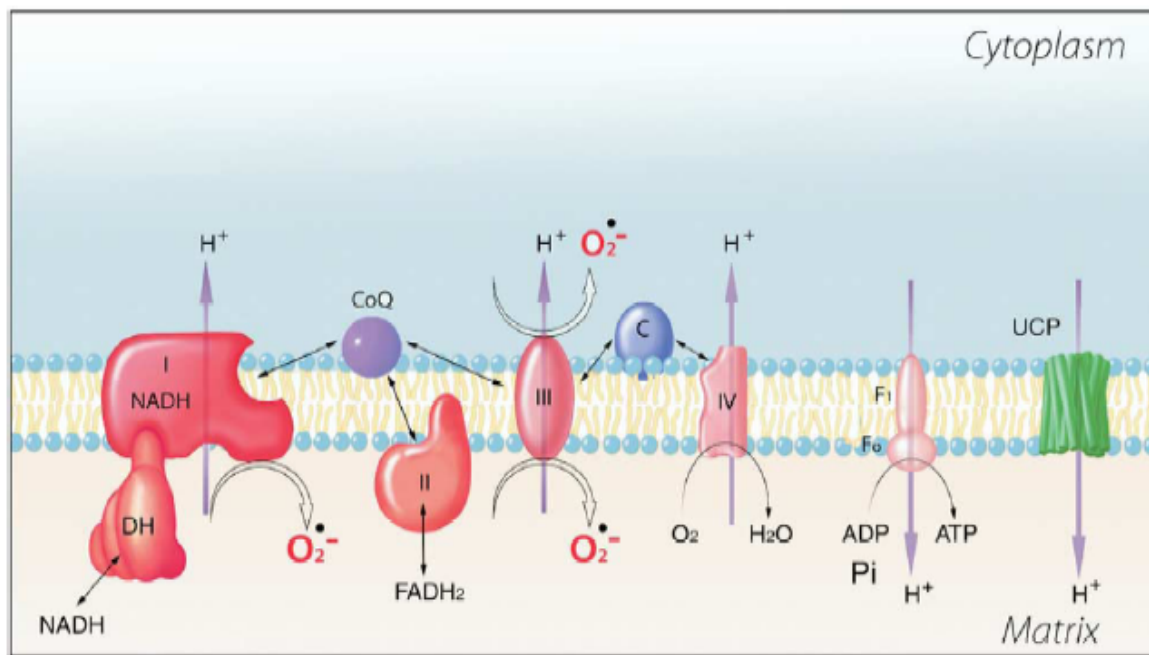
Figure 1.2 A Schematic Model of ROS Generation in the Mitochondria

The four respiratory chain components are shown: complexes I, II, III, and IV, with their substrates, cofactors, and the paths of electron flow. The major production sites of superoxide anions at sites I and III are identified along with the major ROS producing pathways. The different complexes of oxidative phosphorylation are color coded with regard to the magnitude of Eox for reducing oxygen, with red (dehydrogenases [DH] and site I) having the highest potential and site IV the lowest potential. The family of uncoupling protein (UCP) reduces the overall mitochondrial membrane potential ($\Delta\Psi$). This is believed to result in a generalized decrease in Eox for both sites I and III and hence a reduction in ROS formation.

Abbreviations: CoQ: coenzyme Q; NAD: nicotinamide adenine dinucleotide; FAD: flavin adenine dinucleotide; C: cytochrome c1; Fo and F1 : membrane and soluble domains of ATP synthase, with subunits designated.

(From Balaban, R. S., Nemoto, S., and Finkel, T. (2005). "Mitochondria, oxidants, and aging." Cell **120**(4): page 485.)

Complex III as a source of superoxide

The Complex III contributes to $O_2^{\bullet-}$ generation by autooxidation of the ubisemiquinone anion radical ($UQ^{\bullet-}$) either on the inner or outer membranes surfaces (Fig. 1.2). During this process, one-electron reduction of oxygen by $UQ^{\bullet-}$ leads to $O_2^{\bullet-}$ formation (Freeman 1982; Muller, Liu et al. 2004). Within Complex III, the transfer of electrons from ubiquinol (UQH_2) (the reduced form of coenzyme Q) to cytochrome *c* oxidase (and the associated H^+ pumping) is catalyzed in the so-called "Q cycle" (Crofts 2004). This process occurs initially on the matrix side of the membrane, and is the repeated on the intermembrane space face of the membrane (Kowaltowski and Vercesi 1999; Balaban, Nemoto et al. 2005). Under normal conditions, $UQ^{\bullet-}$ is immediately oxidized by cyt *b*L (proximal to the cytosolic side) to UQ. Since O_2 is more soluble in lipid than water (its partition between lipid bilayer and aqueous intermembrane space is 4–5 in favor of the lipid), the O_2 dissolved in the membrane can react with $UQ^{\bullet-}$, especially when electron transport is slowed by high membrane potential ($\Delta\Psi$). This reaction yields superoxide, $O_2^{\bullet-}$. Previous studies have demonstrated that about half of $O_2^{\bullet-}$ produced on the Complex III is released into the matrix (Muller, Liu et al. 2004).

Other possible mitochondrial ROS sources

Complex II (succinate dehydrogenase) is considered as a possible source of ROS. It consists of two subunits embedded in the inner membrane from the matrix side and stabilized by three anchoring proteins. When the enzyme is damaged by the oxidative stress or aging, Complex II produces $O_2^{\bullet-}$, most probably by oxidation of the flavin semiquinone radical formed by reduced flavin in the absence of an immediate electron transfer partner

(Zhang, Yu et al. 1998). In hypoxia situation, the activity of Complex II is switched from the succinate dehydrogenase to the fumarate reductase; this induces one of the most powerful $O_2^{\bullet-}$ sources on the complex I (Paddenberg, Ishaq et al. 2003).

Reduced flavins may generate $O_2^{\bullet-}$ in other flavoprotein enzymes. Two proteins involved in β- oxidation of fatty acids, the electron transfer flavoprotein and the electron transfer flavoprotein-quinone oxidoreductase, produce $O_2^{\bullet-}$ in the matrix (St-Pierre, Buckingham et al. 2002). The dihydrolipoamide dehydrogenase in matrix (Starkov, Fiskum et al. 2004) and 3-phosphoglyceral dehydrogenase in the inner membrane (Drahota, Chowdhury et al. 2002) were also reported to provide $O_2^{\bullet-}$ formation.

Monoamineoxidase, bound to the outer mitochondrial membrane and oxidizing biogenic amine, is another mitochondrial H_2O_2 source (Cadenas and Davies 2000).

1.2.1.2 Endogenous autooxidation compounds

Small Cytoplasmic Molecules

A variety of small soluble molecules in the cellular cytoplasm capable of undergoing oxidation-reduction reactions may produce reactive oxygen species. These include thiols (Baccanari 1978), catecholamines (Halliwell 1999), flavins (Ballou 1969), tetrahydropterins (Fisher 1973) and quinones (Kozlov 1998). Superoxide anion is the primary radical formed by the reduction of molecular oxygen by these molecules. Transition metals, such as Fe^{3+}, can be reduced to Fe^{2+} by thiol and catecholamines; then Fe^{2+} can autoxidize and produce superoxide radical, $O_2^{\bullet-}$ (Freeman 1982; Halliwell 1999).

Hydrogen peroxide, a secondary product of autooxidation, can be generated via spontaneous or enzymatic dismutation of superoxide anion (Freeman 1982).

Cytoplasmic Proteins

Numerous cytoplasmic enzymes generate oxygen free radicals during their catalytic cycling. Xanthine oxidase, aldehyde dehydrogenase and hemoglobin are important examples. Xanthine oxidase regulates the metabolism and elimination of purine bases. Adenine can be degraded to uric acid and spontaneously generate H_2O_2 during the reduction (Freeman 1982; Maritinez-Cayuela 1995). Hemoglobin can be oxidized producing oxygen free radicals, $O_2^{\bullet-}$. A molecule of oxyhemoglobin (haem-Fe^{2+} + O_2) undergoes decomposition and releases $O_2^{\bullet-}$ and methemoglobin (haem-Fe^{3+}) via the action of the methemoglobin reductases (Halliwell 1999).

1.2.1.3 Membrane Enzymes

Respiratory burst

ROS are generated by certain cells of immune systems, such as phagocytic cells (neutrophils, eosinophils and mononuclear phagocytes) and B and T-lymphocytes. Upon activation these cells abruptly increase oxygen consumption, a process known as the respiratory burst. This process is caused by an enzyme named reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase. NADPH oxidase, composed of membrane-associated and cytosolic proteins, is an electron donor that transfers reducing equivalents from NADPH to O_2 and generates $O_2^{\bullet-}$ (reaction 1.8). NADPH oxidase is a complex enzyme composed of five components: two membrane-bound ones are the

gp91^{phox} and p22^{phox} subunits, which comprise flavocytochrome b_{558}, two cytosolic components, p47^{phox} and p67^{phox}, and a Rac-related GTP-binding protein, all thought to be required in oxidase activation. (*phox* means "phagocyte oxidase", p: protein, and gp: glycoprotein). The activation of the oxidase involves two processes: phosphorylation and translocation. Phosphates are incorporated into p47^{phox} and p67^{phox} and then the cytosolic components translocate to the plasma membrane to assemble the active oxidase. As a result, the reactive oxygen intermediates are produced (Chanock 1994; Darley-Usmar 1995; Koner 1997; Longoni 1998; Ferraria 2000; Hessel 2000; Itou 2001; Zusterzeel 2001). The superoxide anion then is catalytically converted to hydrogen peroxide (reaction 1.9).

$$2\,O_2 + NADPH \rightarrow 2\,O_2^{\bullet-} + H^+ + NADP^+ \qquad (1.8)$$

$$2\,O_2^{\bullet-} + 2\,H^+ \xrightarrow{SOD} 2\,O_2 + H_2O_2 \qquad (1.9)$$
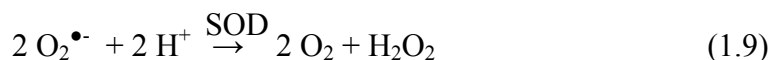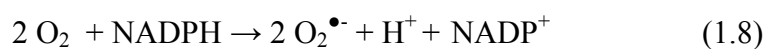
Hydrogen peroxide interacts with myeloperoxidase (MPO), which is contained in neutrophils to produce hypochorous acid, which is a powerful substance involved in killing microorganisms during the immune response.

Microsomal electron transport systems-cytochrome P-450 enzymes

Non-specific monooxygenase, cytochrome P-450 enzyme (CYPs), is membrane bound terminal oxidase present mainly in endoplasmic reticulum. It is a multi-enzyme system, includes FAD/FMN-containing NADPH-cytochrome P450 reductase and cytochrome b_5. In endoplasmic reticulum, CYPs catalyzes a wide variety of substrate reduction reactions. NADPH or NADH is a required cofactor for this reaction. CYPs produce $O_2^{\bullet-}$ and H_2O_2 by autooxidation (Freeman 1982; Kukielka 1995).

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Lipoxygenase and cyclooxygenase

Lipoxygenase and cyclooxygenase are haem-containg dioxygenases catalyzing reactions in the synthesis pathways of leukotrienes, thromboxanes and prostaglandins, which involve oxygen free radical production. However, the ROS can deactivate the cyclooxygenase enzyme, which can be a feedback control of prostaglandin synthesis. Some xenobiotics can be metabolized to more toxic species and then produce very reactive oxygen species by cyclooxygenase catalysis (Maritinez-Cayuela 1995).

1.2.1.4 Peroxisomes

Peroxisomes contain a high concentration of oxidases that use molecular oxygen to remove hydrogen atoms from specific organic substrates in an oxidative reaction that produces cellular hydrogen peroxide. Peroxisomes contain catalase, and H_2O_2-producing-flavin eoxidase such as D-amino acid oxidase and glycollate oxidase which can catalyze molecular oxygen reduction without the formation of the superoxide anion radical (Freeman 1982; Maritinez-Cayuela 1995; Fang 2002). However, excessive $HO^\bullet$ produced by Fenton reaction is able to initiate lipid peroxidation of peroxisomal membrane, resulting in its rupture and release of peroxisomal content into the cytosol (Jezek and Hlavata 2005).

1.2.2 Exogenous sources

Many environmental stimuli could induce high levels of ROS, such as xenobiotics and drugs, ultraviolet (UV) radiation, ionizing radiation, pollution, ultrasound, heat, freezing, and cytokines (Gebicki 2000).

1.2.2.1 Radiation

Formation of radicals by ionizing radiation

Radicals can be generated in both chemical and biological systems by a multitude of different pathways. Radical formation has two categories: direct cleavage of bonds and electron transfer processes. In the first process is the bond between two parts of a molecule is broken so each part of molecule ends up with an unpaired electron. This includes the interaction of high energy radiation, such as γ-radiation, high energy electrons, neutrons and X-ray, with water, the action of high temperature on molecules, and the action of UV and visible light on susceptible molecules. Electrons transfer to or from a molecule results in the generation of a species which very rapidly fragments. Metal-ion or enzymatic-mediated oxidation or reduction of peroxides and also the interaction of some forms of radiation with water can be involved in this process.

Radiation techniques are widely used in radical generation. The radical species formed are the hydrated electron (e^-_{aq}), hydrogen atom ($H^\bullet$), and hydroxyl radicals. Besides, these molecular products are also produced, such as hydrogen (H_2) and hydrogen peroxide (H_2O_2). G values are calculated in terms of the number of atoms or molecules produced per 100eV absorbed or, in SI units, 1J of energy absorbed by the solution. The different radicals can be selectively generated by γ-irradiation of water under different atmospheres (Table 1.3) (Van Sonntag 1987; Hagen 1989; Jonah 1995; Davies 1997).

Table 1.3 ^{60}Co γ-irradiation G values of water radicals, ions and molecular products in deoxygenated and oxygenated solutions

	N$_2$	N$_2$O[+]	N$_2$O/O$_2$*	O$_2$[#]
HO$^\bullet$	2.7	5.4	5.4	2.7
H$^\bullet$	0.55	0.55	-	-
e$_{aq}$-	2.65	-	-	-
HO$_2$$^\bullet$/O$_2$$^{\bullet-}$	-	-	0.55	3.2

[+] N$_2$O-saturated = 2.2×10^{-3} mol dm^{-3}.

* N$_2$O/O$_2$ (4:1 v:v) saturated.

[#] O$_2$-saturated = 2.2×10^{-3} mol dm^{-3}.

(This Table is modified from: Davies, M. J., and Dean, R. T. (1997). <u>Radical-mediated protein oxidation</u>. New York, Oxford University Press Inc. Page 27).

Reaction of hydroxyl radicals

More than 90 % of living cells is made up by water. Exposure of cells to high doses of γ-radiation results in the production of a whole range of radicals and nonradical species from ionization of intracellular water. The effects of ionizing radiation on biomolecules are mainly due to the formation of hydroxyl radicals, aqueous electrons, superoxide radicals and hydrogen peroxide by water radiolysis (Table 1.3). These radicals attack molecules and biomolecules by a variety of reactions, including hydrogen abstraction, electron abstraction by the radical (oxidation of the substrate), and electron donation by the radical (reduction of the substrate), addition (OH-addition), fragmentation, and substitution (addition followed by rapid elimination).

$$\text{H-abstraction: } RH + HO^\bullet \rightarrow R^\bullet + H_2O \qquad (1.10)$$

$$\text{Electron abstraction: } HO^\bullet + Cl^- \rightarrow OH^- + Cl^\bullet \qquad (1.11)$$

Electron donation: $Q^{\bullet -} + O_2 \rightarrow Q + O_2^{\bullet -}$ (1.12)
(Q: is a quinone)

OH-addition: $R' + HO^{\bullet} \rightarrow R^{\bullet}OH$ (1.13)

Fragmentation: $(CH_3)_3CO^{\bullet} \rightarrow {}^{\bullet}CH_3 + CH_3C(O)CH_3$ (1.14)
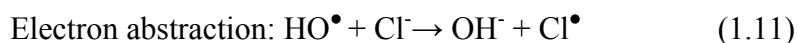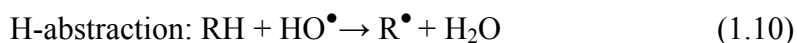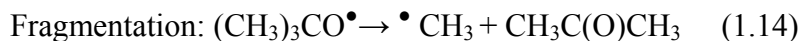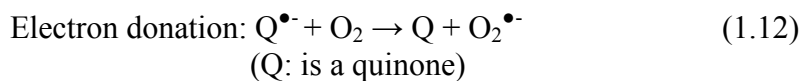
Furthermore, reactions of a radical with a non-radical must give rise to either a further radical species or a species which is oxidized or reduced. In the presence of oxygen, these radicals can be transformed into peroxyl radicals.

$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$ (1.15)

The latter type of the reactions involves the radicals and certain metal ions.

$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$ (1.16)

$HO^{\bullet} + Cu^{1+} \rightarrow OH^- + Cu^{2+}$ (1.17)

The sequence of reduction potential of these radicals is: $O_2^{\bullet -} < HOO^{\bullet}/HOO^- < N_3^{\bullet}/N_3^- < H_2O_2 < HO^{\bullet}/OH^-$ (Hagen 1989; Jonah 1995; Davies 1997).

Gamma radiation technique was used for producing different radicals in my experiments. This technique can provide precisely known nature and yields of radicals made and a huge library exists of the rate constants of their reactions. Different conditions were used to produce exact yields and desired radicals.

1.2.2.2 Other exogenous sources

Air pollutants (cigarette smoke, car exhaust, and industrial contaminants) as well as natural gases (ozone, high concentration of oxygen and hyperbaric oxygen) constitute major sources of ROS which can attack and damage the organism by inducing oxidative stress (Sorensen, Autrup et al. 2003). Drugs are also a major source of ROS (Iinuma, Yoshikawa et al. 1998; Naito, Yoshikawa et al. 1998). For instance, indomethacin whose mechanism of activity is mediated via production of ROS induces lipid peroxidation that plays a crucial role in the development of the gastric mucosal injury (Chinev, Bakalova et al. 1998). A large number of xenobiotics, such as toxins, pesticides, paraquat (Bayol-Denizot, Daval et al. 2000; Valavanidis, Vlahogianni et al. 2006) and chemicals, like alcohol (Muresan and Eremia 1997; Dhindsa, Tripathy et al. 2004) , produce ROS as a by-product of their metabolism *in vivo*. Heat can also induce the formation of ROS which leads to DNA damage (Bruskov, Malakhova et al. 2002). The invasion of pathogenic bacteria and viruses might induce the production of many ROS species by direct release from the invaders or an endogenous response induced by phagocytes and neutrophils (Heiser, Obwald et al. 1998).

1. 3 Antioxidant defense systems

Chemical antioxidants are known as compounds able to inhibit the processes of oxidation. They can inhibit ROS processes at two phases: (1) in the initiation phase: antioxidants remove ROS from the organisms; and (2) in the propagation phase: antioxidants degrade hydroperoxides to inactive products. Antioxidant–type compounds break the sequence of oxidative reactions by reacting with ROS on a single-stage pathway to generate non-reactive products or on a two-stage pathway, where at first a weak-type compound is formed from the antioxidant. This is followed by the weak radicals joining to another (or the same) compound to form a non-reactive complex (Kulikowska-Karpiñska and Moniuszko-Jakoniuk 2004). The list of compounds which can be classified as antioxidants is being constantly enriched. They can be classified into enzymatic and non-enzymatic systems (Fang, Yang et al. 2002).

1.3.1 Enzymatic defense systems

All aerobic ells contain antioxidative enzymes. The four major enzymes are superoxide dismutase, catalase, glutathione peroxidase and, glutathione reductase.

1.3.1.1 Superoxide dismutase (SOD)

In 1969, SOD was first discovered for the antioxidant ability which catalyses dismutase reactions of the superoxide anion radicals and leads to less-reactive hydrogen peroxide generation (reaction 1.18) (McCord 1969). The reaction catalyzed by SOD is extremely efficient, limited in essence only by diffusion (Nordberg and Arner 2001).

$$2\ O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{1.18}$$

In eukaryotic cells, two metal-containing SOD isoenzymes are known, differing in cofactors, sensitivity to inhibitors and location. Manganese-containing superoxide dismutase (Mn-SOD), an 80-kDa tetramer, is located in the mitochondrial matrix. Mn-SOD contains manganese at its active site. In mitochondria, superoxide is formed in relatively high concentrations due to the leakage of electrons from respiratory chain. The Mn-SOD is obviously essential. It is also one of the most effective antioxidant enzymes that have antitumor activity. Behrend and his colleagues discovered that overexpression of Mn-SOD leads to tumour growth retardation in different cell lines (Behrend, Henderson et al. 2003).

Cytosolic superoxide dismutase (Cu, Zn-SOD), a 32-kDa dimer, is made by two identical protein subunits, each with an active site containing one copper and one zinc ion (Schnackenberg 2002). Zinc is responsible for subunit stability, while copper for activity which neutralizes the superoxide anion radicals. Copper ions can be found in two stages of oxidation, as cuprous (Cu^+) and cupric ions (Cu^{2+}) (reaction 1.19 and 1.20). Cu, Zn-SOD plays a major role in the first line of antioxidant defence.

$$SOD\text{-}Cu^{2+} + O_2^{\bullet -} \rightarrow SOD\text{-}Cu^+ + O_2 \qquad\qquad (1.19)$$

$$SOD\text{-}Cu^+ + O_2^{\bullet -} + 2H^+ \rightarrow SOD\text{-}Cu^{2+} + H_2O_2 \qquad\qquad (1.20)$$
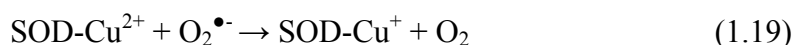
The rate of SOD-catalyzed hydrogen peroxide formation ($k = 2.4 \times 10^9$ $M^{-1}s^{-1}$) from superoxide anion is 10^4 higher than spontaneous dismutation at physiological pH (Martinez-Cayuela 1995; Halliwell 2003).

Unlike intracellular superoxide dismutase, extracellular superoxide dismutase (EC-SOD) is a tetrameric glycoprotein which contains one copper and zinc of each subunit. Most EC-SOD is bound to cell surfaces by association with cell surface carbohydrates. They have a high affinity for certain glycosaminoglycans such as heparin and heparin sulphate. EC-SOD is located in the interstitial spaces of tissue and in extracellular fluids such as plasma, lymph and synovial fluid in humans. Therefore, they are most likely regulated by cytokines in mammalian tissues, rather than by response of individual cells to oxidants (Mates, Perez-Gomez et al. 1999; Valko, Rhodes et al. 2006). EC-SOD can eliminate the interaction of superoxide and nitric acid to from peroxynitrite (Halliwell 2003).

1.3.1.2 Catalase

Catalase, a heme-protein, is widely contributed in nearly all aerobic cells, especially in peroxisomes. It contains four iron atoms per mole of protein. It can protect the cell from the toxic effects of hydrogen peroxide by catalyzing its decomposition into molecular oxygen and water without the production of free radicals (reaction 1.21). Catalase has one of the highest turnover rates of all enzymes; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second (Nordberg and Arner 2001).

$$H_2O_2 + H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2 \tag{1.21}$$
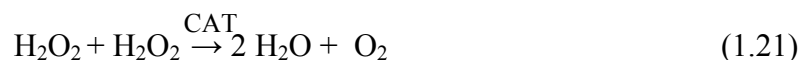
Catalase can also utilize different substrates, such as phenols, methanol, formic acid and ethanol, via coupled reduction of hydrogen peroxide (Mates, Perez-Gomez et al. 1999). Catalase can also lower the risk of hydroxyl radical formation from H_2O_2 via Fenton

reaction catalyzed by Cu or Fe ions (Betteridge 2000). Catalase binds NADPH, which protects the enzyme from inactivation and increases its efficiency (Kirkman and Gaetani 1984; Kirkman, Rolfo et al. 1999).

1.3.1.3 Glutathione peroxidase

Glutathione peroxidase is a selenium-containing tetrameric glycoprotein, that is, a molecule with four selenocysteine amino acid residues. As the integrity of the cellular and subcellular membranes depends heavily on glutathione peroxidase, the antioxidative protective system of glutathione peroxidase itself depends heavily on the presence of selenium. Two forms of the enzyme glutathione peroxidase exist, one is selenium-independent and the other is selenium-dependent. They differ in the number of subunits, the bonding nature of the selenium at the active centre and their catalytic mechanism (Mates, Perez-Gomez et al. 1999).

There are four different Se-dependent glutathione peroxidases in human tissues (GPx1-4). All GPx enzymes can add two electrons to reduce peroxides by forming selenoles (Se-OH) (Valko, Rhodes et al. 2006). The protein is made up of four subunits, each containing one atom of selenium covalently bound to cysteine. Selenium is essential for the catalytic action of the enzyme, which catalyzes the reaction between hydrogen peroxide and glutathione (GSH) to produce the oxidized form of glutathione (GSSG) (reaction 1.22 and 1.23). Glutathione peroxidase is located mostly in the cytosol (70 %), but also in the mitochondrial matrix (20 %) and in the nucleus (10 %). GPx1 and GPx4 are most abundant cytosolic enzymes in most tissues (Nordberg and Arner 2001). As part of the GPx system,

these enzymes eliminate peroxides as potential substrates for Fenton reaction. For instance, these enzymes catalyze the reduction of some organic peroxides, such as lipid peroxides, which makes them important in detoxification (Kulikowska-Karpiñska and Moniuszko-Jakoniuk 2004). To conclude, GPx is a major source of protection against low levels of oxidative stress (Mates, Perez-Gomez et al. 1999).

$$H_2O_2 + 2\ GSH \rightarrow GSSG + 2\ H_2O \tag{1.22}$$

$$LOOH + 2\ GSH \rightarrow GSSG + LOH + 2\ H_2O \tag{1.23}$$
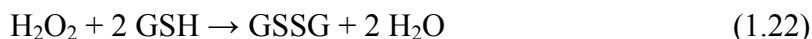
1.3.3.4 Glutathione reductase

Glutathione reductase is an enzyme which reduces oxidized glutathione using NADPH and contributes in the GRx system. NADPH undergoes regeneration associated with enzymes, including glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase, which catalyze oxidation, with the NADP as a co-substrate (Martinez-Cayuela 1995; Dreher and Junod 1996).

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+ \tag{1.24}$$

1.3.2 Non-enzymatic defense systems

Low-molecular weight antioxidants play a major role in protection from oxidative stress. Many of them show evidence of preventive and interceptive action. They can either inhibit oxidation by reacting with oxidative factor or by reacting with indirect products of oxidation. Low-molecular antioxidants are divided into two classes: hydrophilic (ascorbic acid, glutathione, uric acid, flavonoids, creatinine) and hydrophobic (tocoperols, bilirubin, carotenoids) (Jamieson 1989; Dreher and Junod 1996). This section is focused on the hydrophilic antioxidants, especially ascorbic acid, glutathione and flavonoids.

1.3.2.1 Ascorbic acid –Vitamin C

Ascorbic acid (Vitamin C) is an essential micronutrient for many biological and biochemical functions. Humans cannot synthesize ascorbic acid because of lacking the enzyme, gulonolactone oxidase, which catalyzed the last enzymatic step in biosynthetic pathway (Rumsey and Levine 1998). Ascorbic acid has been studied for a variety of chemical, biological and biochemical functions (Martinez-Cayuela 1995; Dreher and Junod 1996; Fang, Yang et al. 2002). Ascorbic acid is an unstable substance, sensitive to heating, particularly in the presence of oxygen and heavy metals (iron and copper). It is stable in ferric ion–free water. It degrades fast in alkaline or neutral solutions. The levels of ascorbic acid found *in vivo* are 30-100μM in human plasma (Carr and Frei 1999; Padayatty, Katz et al. 2003).

Ascorbic acid acts as an electron donor in many intracellular and extracellular reactions. It is a specific electron donor for eight enzymes. These enzymes are involved in collagen hydroxylation, carnitine biosynthesis, norepinephrine synthesis, peptide hormone synthesis, and tyrosine metabolism (Levine, Rumsey et al. 1999; Duarte and Lunec 2005).

Moreover, ascorbic acid has the ability to regulate factors that may influence gene expression, apoptosis and other cellular functions. Many studies indicate that ascorbic acid protects against cell death triggered by various stimuli and a major proportion of this protection is caused by its antioxidant ability. Ascorbic acid plays a role in modulation of the immune systems due to its anti-apoptotic ability. In recent studies, ascorbic acid was found to regulate the AP-1 (activator protein-1) complex, including the Fos and Jun

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superfamilies. Ascorbate treated cells exposed to UV-B irradiation led to a 50 % decrease in JUK (c-Jun N-terminal kinase) phosphorylation which activated AP-1, therefore inhibiting the JUK/AP-1 signalling pathways (Catani, Rossi et al. 2001).

Ascorbic acid is believed to reduce the risk of certain oxidative stress-related diseases, such as hypertension (Schneider, Delles et al. 2005; Vasdev and Gill 2005), atherosclerosis (Lee, Folsom et al. 2004), cataract (Devamanoharan, Henein et al. 1991; Varma, Devamanoharan et al. 1991) and neoplastic diseases (Naidu 2003; Salnikow and Kasprzak 2005).

Ascorbic acid is an endiol form of δ-lactone of 3-keto-gulonic acid. Based on the endiol group of this acid molecule, it has strong reductive properties which are referred to as the major antioxidative factor in aqueous solutions (Kulikowska-Karpiñska and Moniuszko-Jakoniuk 2004). Ascorbic acid can be reversibly oxidized with the loss of one electron to form the free radical, ascorbate free radicals (Asc$^\bullet$), which is further oxidized to dehydroascorbic acid (DHA). Dehydroascorbic acid can be reduced to ascorbic acid via the same intermediate radical, or the ring structure of dehydroascorbic acid can be irreversibly hydrolyzed to yield diketogulonic acid (Fig. 1.3). The latter can be degraded further to form oxalate, threonate, xylose, xylonic acid, and lynxonix acid (Buettner 1988; Togashi, Shinzawa et al. 1994; Nappi and Vass 1997; Pfeffer, Casanueva et al. 1998; Cho, Yang et al. 1999; Zhang and Omaye 2000; Chung, Chung et al. 2001 ; Huang, Liu et al. 2002; Padayatty, Katz et al. 2003).

Ascorbic acid is an outstanding antioxidant for several reasons. First, both ascorbate and ascorbate free radicals have low reduction potentials and can react with most other biologically relevant radicals and oxidants. Second, the ascorbate free radical has a low reactivity due to resonance stabilization of unpaired electron and readily dismutates to ascorbate and dehydroascorbic acid. In addition, ascorbate can be regenerated from both ascorbate free radicals and DHA by enzyme–dependent and independent pathways. The enzymes which can reduce ascorbate free radicals are the NADH-dependent semihydroascorbate reductase and the NADPH-dependent selenoenzyme thioredoxin reductase. For non-enzymatic pathway, DHA can be reduced back to ascorbate by GSH and lipoic acid (Rumsey and Levine 1998; Carr and Frei 1999; Arrigoni and De Tullio 2002).

Ascorbate acid exists in two forms, the reduced form and the oxidized form. They are transported across cell membrane. The transport of ascorbate acid is Na^+-dependent and metabolic energy is required, but transport of DHA is Na^+-independent and metabolic energy is not required in tissues of animals and humans. However, the protein responsible for ascorbate acid transport has not yet been isolated. When ascorbate acts as an antioxidant or enzyme cofactor, it becomes oxidized to DHA. DHA can be used to regenerate ascorbate, directly and indirectly, and also to change the redox state of many other molecules (Fig. 1.4) (Rumsey and Levine 1998). Many cells have been shown capable of using extracellular DHA to produce intracellular ascorbate, including adipocytes, astrocytes, endothelial cells, erythrocytes, granulose cells, hepatocytes, neutrophils, osteoblasts and smooth muscle cells (Wilson 2005).
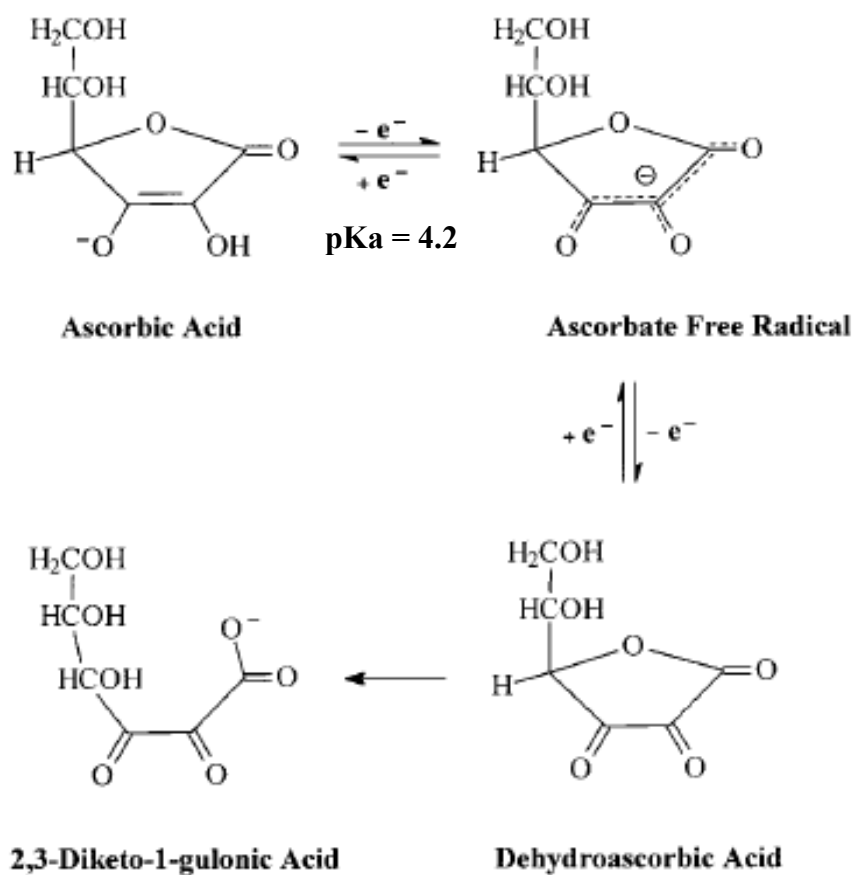
Figure 1.3 Redox metabolism of ascorbic acid

(Figure is from May, J. M. (1999). "Is ascorbic acid an antioxidant for the plasma membrane?" <u>FASEB J</u> **13**(9): page 996).

Of particular interest for this work is the enhancement of ascorbate concentration in the culture cells, HL-60 cells. In this cell line, DHA entry involves uptake via a facilitated-diffusion mechanism and rapid reduction to ascorbic acid intracellularly. The chemical structure of DHA is similar to glucose; therefore, DHA competes with glucose for uptake through several isoforms of facilitative glucose transporters (GLUT 1 and GLUT 3) (Liang, Johnson et al. 2001; Wilson 2002). Intracellular dehydroascorbic acid is immediately reduced to ascorbate via the glutathione-dependent pathway (Guaiquil, Farber et al. 1997). The uptake and reduction of DHA in neutrophils is show in Figure 1.4, which contains a minor error: instead of hypochlorous acid (HOCl), the diagram listed perchloric acid ($HOCl_4$) as a product of reaction of hydrogen peroxide with NaCl in reaction catalysed by myeloperoxidase.
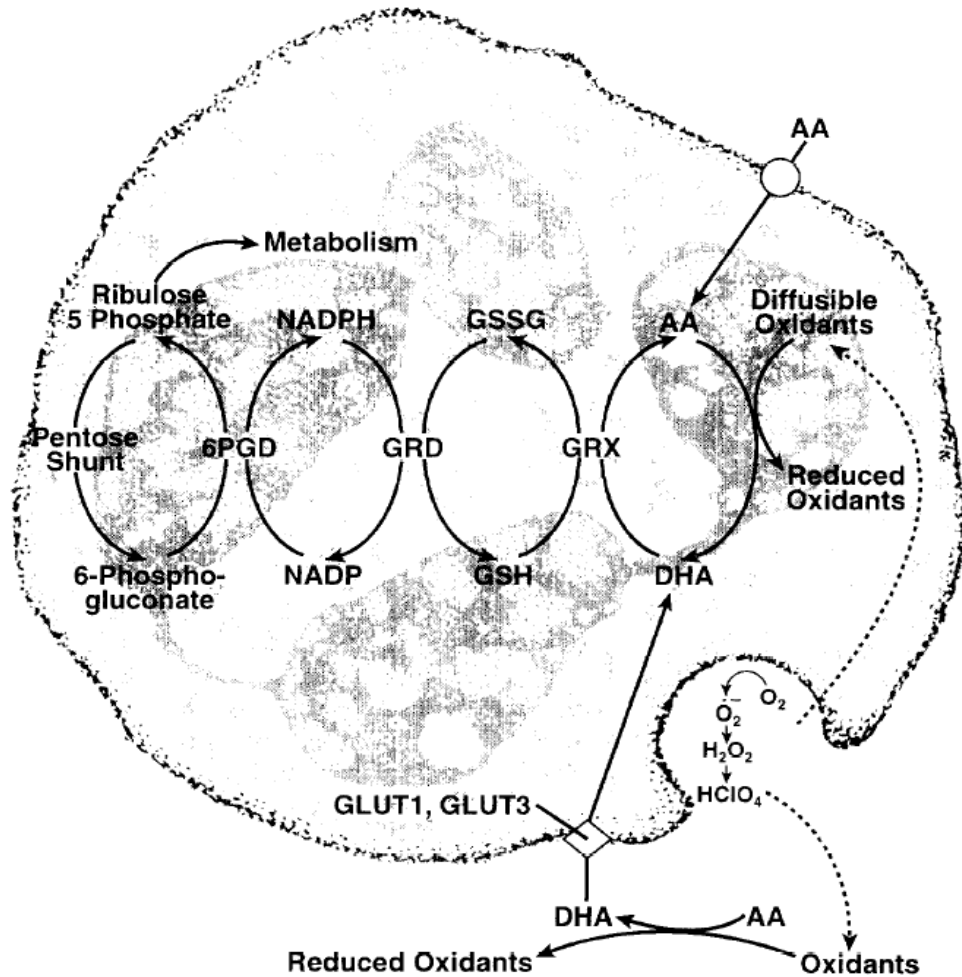
Figure 1.4 A model of dehydroascorbic acid and ascorbate transport and recycling in human neutrophils

Ascorbate and dehydroascorbic acid are transport differently. The putative ascorbate transporter (Open circle) transports ascorbate and probably maintains mM concentrations of ascorbate inside neutrophils. The ascorbate transport protein has not been isolated. With activation, neutrophils secrete reactive oxygen species, which oxidize extracellular ascorbate to dehydroascorbic acid. Dehydroascorbic acid is rapidly transported by glucose transporter isoforms GLUT1 and GLUT3 (open diamond). Intracellular dehydroascorbic acid is immediately reduced to ascorbate. The proposed mechanism of reduction could require glutathione, NADPH, and the enzyme shown. Abbreviations: AA, ascorbate; DHA, dehydroascorbic acid; GRX, glutaredoxin; GSH, reduced glutathione; GSSH, oxidized glutathione; 6-PGD, 6-phosphogluconate dehydrogenase; GRD, glutathione reductase. (Figure is from Rumsey, S. C. and M. Levine (1998). "Absorption, transport, and disposition of ascorbic acid in humans." <u>Nutritional Biochemistry</u> **9**: page 120.)

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1.3.2.2 Glutathione

Glutathione (L-γ-glutamyl-L-cysteinylglycine), a tripeptide, is a compound soluble in water and is involved in the antioxidant cellular defense system by the thiol (-SH) of cysteine residue (Fig. 1.5) (Nordberg and Arner 2001; Kulikowska-Karpiñska and Moniuszko-Jakoniuk 2004). GSH is a multifunctional intracellular non-enzymatic antioxidant. It is also the most abundant non-protein sulphur molecule in the cells and has a number of physiological roles. GSH is synthesized intracellularly from cysteine, glutamic acid and glycine via two sequential ATP-consuming steps, which are catalysed by γ-glutamylcysteine synthetase (GCS) and GSH synthetase (Wu, Fang et al. 2004).
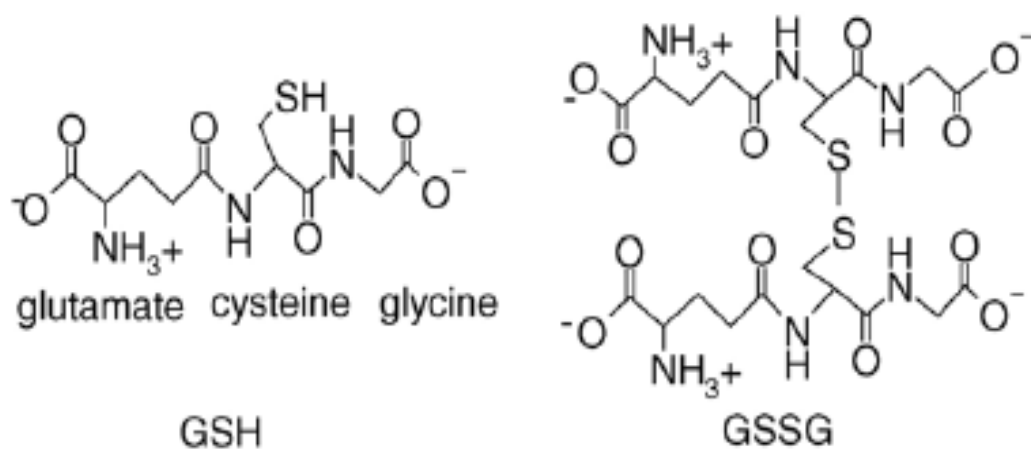
Figure 1.5 Structures of reduced (GSH) and oxidized (GSSG) glutathione

(From: Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M. and Mazur, M. (2006). "Free radicals, metals and antioxidants in oxidative stress-induced cancer." <u>Chem Biol Interact</u> **160** (1): page 24).

Glutathione is be synthesized from the amino acids; L-cysteine, L-glutamate and glycine. The intracellular level of GSH in mammalian cells is in the millimolar range, whereas micromolar concentration is found in plasma (Pastore, Federici et al. 2003). In cells, glutathione occurs at high concentrations in the cytosol (1-11 mM), mitochondria (5-11 mM) and nucleus (3-15 mM); its concentration is lower in the endoplasmic reticulum (2 mM) (Valko, Rhodes et al. 2006). In mitochondria, glutathione plays the major role in the cell protection against physiological oxidative stress (Balendiran, Dabur et al. 2004) .

Glutathione can be free or bound to proteins. Over 90 % of glutathione occurs in cells in a reduced form (GSH) and only a small per cent in an oxidized form (disulfide, GSSG) (Bartosz 1993; Hancock, Desikan et al. 2003). The reduced form of glutathione can be readily converted non-enzymatically to oxidized form by free radicals and reactive oxygen species, and can be reverted to the reduced form by the action of the enzyme glutathione reductase. The cell redox status depends largely on the ratio of the reduced and oxidized forms of glutathione (GSH/GSSH) and appears to be an indicator of the cellular redox state in cell. The ratio is >10 under normal physiological conditions (Wu, Fang et al. 2004).

Glutathione is a nucleophilic scavenger and an electron donor via the sulfhydryl group of its residue, cysteine. The reaction of GSH with the radical $R^\bullet$ can be described:

$$GSH + R^\bullet \rightarrow GS^\bullet + RH \tag{1.25}$$

The thiyl radical (GS$^\bullet$) then dimerise in cells to form the non-radicals product, oxidized glutathione (GSSG) (reaction 1.26) or a variety of other less reactive products, depending on conditions (Wardman 1988).

$$GS^\bullet + GS^\bullet \rightarrow GSSH \qquad\qquad (1.26)$$

GSSG is accumulated inside the cells, the ratio of GSH/GSSG is good measure of oxidative stress of an organisms.

The main protective roles of GSH against oxidative stress have been described. Glutathione is the cofactor of several antioxidant enzymes such as glutathione peroxidase (GPx) and glutathione reductase. Glutathione peroxidase (selenium-containing) detoxifies peroxides, such as hydrogen peroxide and other peroxides, to H_2O. Glutathione reductase contains flavin adenine dinucleotide and uses reducing equivalents from NADPH to convert GSSG to 2 × GSH (Fig. 1.4) (Raggi, Mandrioli et al. 1998). GSH also participates in amino acid transport through the plasma membrane.

GSH can scavenge hydroxyl radicals and singlet oxygen directly and provide the first line of defence against ROS. GSH-dependent enzymes provide the second line of defence, as they primarily detoxify noxious by-product generated by ROS and also help to prevent propagation of free radicals. Another antioxidant activity of glutathione is the maintenance of the antioxidant/reducing agent ascorbate in its reduced state (Shang, Lu et al. 2003). This is accomplished via glutathione-dependent dehydroascorbate reductase which is comprised of glutaredoxin and the protein isomerase reductase.

Glutathione may also react with the reactive nitrogen species peroxynitrite to form *S*-nitrosoglutathione. Glutathione S-transferases (GSTs) consist of a family of multifunctional enzymes that metabolize a wide variety of electrophilic compounds via glutathione conjunction. GSTs are involved in the detoxification of xenobiotic compounds and in the protection against such degenerative diseases as cancer (Masella, Di Benedetto et al. 2005). The mechanism of these enzymes involves a nucleophilic attack by glutathione on an electrophilic substrate. The resulting glutathione conjugates that form are more soluble than the original substrates and thus more easily exported from the cell. The release of glutathione-S-conjugates from cells is an ATP-dependent process mediated by membrane. These processes lower the level of total intracellular glutathione. Therefore, in order to maintain a constant intracellular GSH concentration, the GSH consumed has to be recovered by resynthesis from its constituent amino acid (Pastore, Federici et al. 2003; Masella, Di Benedetto et al. 2005).

GSH regulates redox signalling by alternations in both the level of total GSH and in the ratio of its oxidised (GSSG) to reduced (GSH) forms (Jones, Carlson et al. 2000). Cellular GSH depletion has been reported to be associated with decreased cell proliferation in endothelial cells and increased proliferation of fibroblasts. GSH also participate in regulating activation of various transcription factors, such as nuclear factor NF-κB and activator protein AP-1. GSH protect cells against apoptosis via multifactorial mechanisms involved in detoxification and modulation of redox state and the subsequent redox-sensitive signalling pathways and interaction with pro-and anti-apoptotic signals (Masella, Di Benedetto et al. 2005).

GSH depletion is associated with a number of disease states in humans, such as in the inherited deficiencies of the GSH-synthesising enzymes, where individuals reveal limited or generalised GSH deficiency, in conditions such as haemolytic anaemia, spinocerebellar degeneration, peripheral neuropathy, myopathy and aminoaciduria, and often in development of severe neurological complications and cancer (Anderson 1997).

1.3.2.3 Flavonoids

Flavonoids are a family of low molecular–weight polyphenolic antioxidants found ubiquitously in fruits and vegetables as well as in food products and beverages derived from plants such as olive oil, tea, and wine juice (Ishige, Schubert et al. 2001). Over 4000 structurally unique flavonoids have been identified and can be subdivided into 13 classes. The flavonoids have multiple functions including anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities (Middleton, Kandaswami et al. 2000).

Due to their chemical nature , flavonoids act as potent metal chelators and free radical scavengers (Bors, Heller et al. 1996). Flavonoids have remarkable chain-breaking antioxidant properties and play a vital biological role in the function of scavenging free radicals (Rice-Evans, Miller et al. 1997). ROS that can be scavenged or whose formation can be inhibited by flavonoids, include superoxide anion, hydrogen peroxide, singlet O_2 and perhydroxyl, hydroxyl, alkoxyl and peroxyl radicals (Middleton, Kandaswami et al. 2000; Rojkind, Dominguez-Rosales et al. 2002).

The hydroxyl groups of radicals attached to the aromatic ring structures of flavonoids enable the flavonoids to undergo a redox reaction that helps them to scavenge free radicals more easily. They have a stable delocalization system, consisting of aromatic and heterocyclic rings as well as multiple unsaturated bonds, which helps to delocalize the resulting free radicals. The presence of certain structural groups of flavonoids, allow forming transition metal-chelating complexes that can regulate the production of reactive oxygen species such as $O_2^{\bullet-}$ and $HO^\bullet$ (Peng, Strack et al. 2003).

Rutin (Vitamin P) (Milde, Elstner et al. 2004), silibinin (Bhatia, Zhao et al. 1999), trolox (Wei, Zhou et al. 2006), epigallocatechin gallate (Graham 1992) and gallic acid (Soong and Barlow 2006) are compounds to be screened in this study for their ability to protect protein from oxidative ROS.

1.3.2.4 Nitroxides

Nitroxides are synthetic, small stable organic free radicals with an unpaired electron on the substituted heterocyclic ring ($N-O^\bullet$). Nitroxides attenuate oxidative damage by several mechanisms, including oxidation of reduced transition metal ions, detoxification of intracellular radicals such as alkyl, peroxyl, alkoxyl, hydroxyl, superoxide, and thiyl (Damiani, Kalinska et al. 2000), and dismutate superoxide anion radicals in a catalytic fashion mimicking the of superoxide dismutase (SOD) with rate constants in the range of 10^6-10^7 $M^{-1}s^{-1}$(Xavier, Yamada et al. 2002) . Nitroxides participate in redox reactions with a variety of oxidants and yield hydroxylamines and oxo-ammmonium cations. Both of the nitroxide and hydroxylamine functions are antioxidants. These two antioxidants are

recycled by the nitroxide reduction and reoxidation (enzymatic or non-enzymatic) (Zhang, Goldstein et al. 1999).

Nitroxides vary in size, structure, charge, and lipophilicity. Two nitroxides, 2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO) and it's hydroxylated derivative, 4-hydroxy-2 2,6,6-tetramethylpiperidine-1-oxy (TEMPOL) (see Fig. 1.6), have similar antioxidant abilities. They can protect DNA in cells, inhibit growth of cancer cells, protect rats from the effects of cerebral injury, inhibit protein oxidation, induce apoptosis in neoplastic cells, decrease obesity and tumour incidence in mice, protect cells from γ radiation and H_2O_2 oxidation, and inhibit oxidative damage to cardiomyocytes (Zhang, Goldstein et al. 1999; Damiani, Kalinska et al. 2000; Xavier, Yamada et al. 2002; Samuni and Barenholz 2003; Piehl, Facorro et al. 2005).
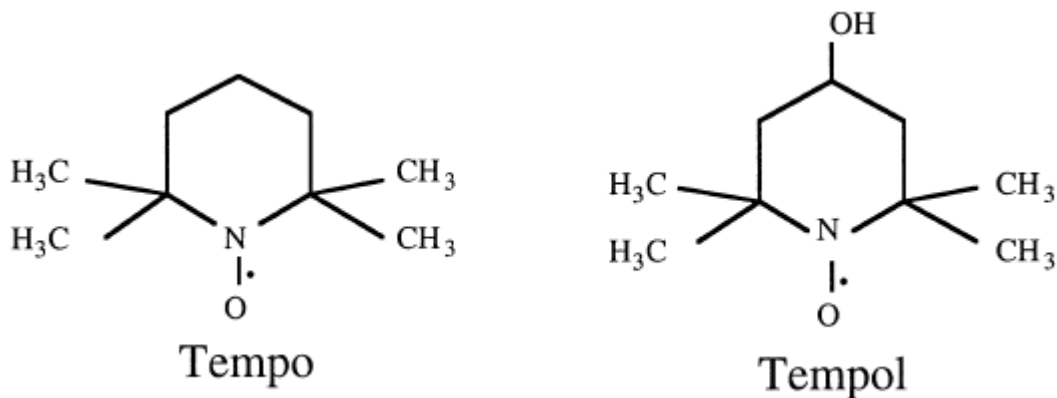
Figure 1.6 Structures of nitroxide compounds

(From Samuni, A. M., and Barenholz, Y. (2003). "Site-activity relationship of nitroxide radical's antioxidative effect." Free Radic Biol Med **34**(2): page 178.)

1.3.2.5 Nitrones

Nitrones ($\equiv$ N$^+$-O$^-$) are commonly used in free radical trapping in different chemical and biochemical reactions. This function is based on the ability of the nitrone group (1) to add short-lived free radicals to form more stable nitroxide radicals (2) (see Fig. 1.7) (Barlukova, Gritsan et al. 2005). Therefore, nitrones are widely as spin traps for EPR detection of short-lived radical intermediates in biological systems (Potapenko, Bagryanskaya et al. 2004). The nitrone-based free radical spin traps, especially α-phenyl-N-tert-butyl nitrone (PBN) (Paucard, Besson et al. 2005) and 5, 5-dimethyl-1-pyroline-1-oxide (DMPO) (Potapenko, Bagryanskaya et al. 2004) are most widely used. Especially PBN, which appears to protect against hyperoxia-induced NO toxicity by anti-inflammatory action (Paucard, Besson et al. 2005), provides protection against AChE inhibitor-induced oxidative stress mainly by preventing seizures (Gupta, Milatovic et al. 2001), and has neuroprotective effects due to its free radical scavenging activity (Milivojevic, Babic et al. 2001). PBN can also act as an inhibitor of ROS-induced age-associated damage in biological system (Carney and Floyd 1991).
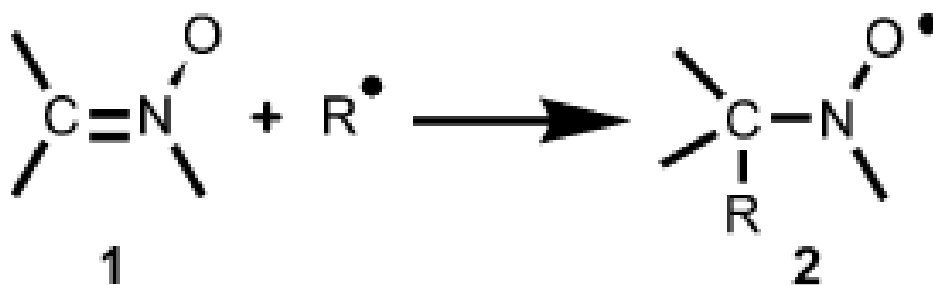
Figure 1.7 Spin trapping reaction of nitrones

(From: Barlukova, et al. (2005). "OD ESR detection of the radical anions of cyclic nitrones in liquid solutions" Chemical Physics Letters **401**(1-3): page 63.)

1. 4 Reactive oxygen species and human diseases

Reactive oxygen species generated during metabolism, when uncontrolled, can initiate processes leading to clinical manifestations (Tsai, Hung et al. 1998). The effects include peroxidative changes in membrane and other cellular components, such as DNA, protein and lipids. Excess ROS can initiate oxidative chain reactions and peroxidation (Table 1.4) (Miller, Brzezinska-Slebodzinska et al. 1993). The link between oxidative stress and pathology is now firmly established and is supported by the identification of over 50 diseases and debilitating conditions it can cause or aggravate, including malnutrition, arthritis, some forms of cancer, asbestosis, diabetic cataracts, atherosclerosis, pulmonary disorders, immune injury, damage to the nervous system (Parkinson's, Alzheimer's), iron overload and many others. An increasingly important condition is the general debility associated with aging, widely believed to be assisted by accumulation of ROS-induced damage over a lifetime (Mates, Perez-Gomez et al. 1999). A selection of the mechanisms of associated with oxidative stress shown in Table 1.4.

Table 1.4 Initiation and propagation of reactive oxygen metabolites

Reaction	Product
$O_2 + e^- \rightarrow O_2^{\bullet-}$	Superoxide
$2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + HOOH$	Hydrogen peroxide
$2O_2^{\bullet-} + Fe^+ \rightarrow O_2 + Fe^{2+}$	Reduced iron
$HOOH + Fe^{2+} \rightarrow O_2 + Fe^{2+} + HO^{\bullet}$	Hydroxyl radical
$HO^{\bullet} + LH$ or $RH \rightarrow H_2O + L^{\bullet}$ or $R^{\bullet}$	Fatty acid or other organic molecule oxidized
$R^{\bullet} + LH \rightarrow RH + L^{\bullet}$	Oxidized fatty acid
$L^{\bullet}$ or $R^{\bullet} + O_2 \rightarrow LO_2^{\bullet}$ or $RO_2^{\bullet}$	Peroxyl radical
$LO_2^{\bullet}$ or $RO_2^{\bullet} + LH$ or $RH \rightarrow LOOH$ or $ROOH$	Lipid peroxide or organic molecule peroxides (protein peroxide)

(From Miller, J. K., Brzezinska-Slebodzinska, E., and Madsen, F. C. (1993). "Oxidative stress, antioxidants, and animal function." <u>J Dairy Sci</u> **76**(9): page 2815.)

Some of the pathologies initiated or aggregated by free radicals and other ROS are listed below.

1.4.1 Aging

In 1956, Denham Harman proposed that free radicals produced during aerobic respiration cause cumulation of oxidative damage, resulting in aging and death. It is called the free-radical theory of aging (Harman 1956; de Magalhaes and Church 2006). The theory states that the aging process results from the accumulated damage caused by reactive oxygen species, highly reactive molecules that are normal by-products of cellular metabolism (Harman 1981; Beckman and Ames 1998). It is now established that aging and age-related diseases are associated with ROS-mediated oxidative damage of lipid, protein, and nuclear

and mitochondrial DNA molecules. With age, antioxidant defences fail to scavenge all potentially damaging radical species. Therefore, the concentration of damaged proteins, lipids and DNA has been reported to increase with age (Bandyopadhyay, Das et al. 1999; Schoneich 1999). The superoxide and hydrogen peroxide induced mutations in mitochondrial DNA and also lead to the formation of defective enzymes which result in generate more ROS to cause further oxidative damage (Reiter, Tang et al. 1997). The hydroxyl and peroxyl radicals cause oxidative damage of proteins resulting aging and age-related degenerative disease (Hensley and Floyd 2002).

1.4.2 Inflammation/ infection

Inflammatory reactions induce the production of reactive oxygen species (ROS) and vice versa. In 1981, Weitzman and Stossel discovered that inflammatory phagocytes produce large quantities of the superoxide anion and hydrogen peroxide which can cause injury to target cells that might contribute to cancer development. Cells in inflammatory conditions or infections were reported to have elevated levels of 8-OH-dG and other DNA damage (Weitzman and Gordon 1990).

Chronic inflammation and the oxidative stress have been closely linked to the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA) (Droge 2002) and systemic lupus erythematosis (SLE) (Evans, Dizdaroglu et al. 2004). The radicals produced in these diseases not only induce tissue damage but also the modification of cellular biomolecules, such as DNA. Elevated DNA levels of 8-OH-dG have been reported in lymphocytes from patients with RA and SLE. A decreased level of intracellular GSH was also detected in T

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cells isolated form the synovial fluid in RA patients (Droge 2002). The lymphocytes from these patients also displayed increased sensitivity to hydrogen peroxide–induced cytotoxicity (Bashir, Harris et al. 1993).

HIV infection is associated with progressive deterioration of the immune systems, leading eventually to lethal opportunistic infections (Mates, Perez-Gomez et al. 1999; Droge 2002). HIV-infected patients show abnormally low plasma cysteine levels (Droge and Holm 1997) and low intracellular GSH levels in peripheral blood lymphocytes (Eck, Gmunder et al. 1989), suggesting an association with oxidative stress.

1.4.3 Ischaemia-reperfusion

Ischaemia and reperfusion can lead to tissue injury and are serious complications in transplantation surgery, haemorrhagic or septic shock, myocardial infraction and stroke (Goode, Webster et al. 1994; Gersh 1998; Evans, Dizdaroglu et al. 2004).

During the period of ischaemia, blood glucose falls; glycolysis and oxidative phosphorylation rate decrease, and subsequently ATP stores are consumed. This alteration leads to xanthine dehydrogenase being converted to xanthine oxidase, which can result in massive of ROS production. The enzyme transfers the electrons coming form substrate to oxygen, rather than NAD^+, leading to the formation of superoxide and then hydrogen peroxide (Granger 1988). Besides, due to hypoxanthine accumulation in response to ischaemia, there is a burst of xanthine oxidase activity with a subsequent potential for generating ROS and hence DNA damage. In the end, the activity of two antioxidant

enzymes such as catalase and SOD are decreased following ischaemia (Homi, Freitas et al. 2002). Thus, as soon as oxygen is reperfused, all these conditions act synergistically to produce high level of ROS in the tissues that have undergone ischaemia.

Several studies reported that the level of DNA damage is raised in post-ischaemia-reperfusion (Evans, Dizdaroglu et al. 2004). In pig, it is clear that ischaemia-reperfusion induces a considerable oxidative stress following transplantation. The level of urinary 8-OH-dG is elevated (Loft, Larsen et al. 1995). In rat, urinary levels of thyroglobulin (Tg) were significantly increased after liver transplantation (Ichikawa, Watanabe et al. 1996). Based on the animal models, the urinary assays are useful for making non-invasive assessments of oxidative stress in the case of patients with ischaemia-reperfusion following organ transplantation (Gelfand, Podnos et al. 2002).

1.4.4 Atherosclerosis

Atherosclerosis is a multifactorial disease characterised by a local thickening of the inner coat of the arterial wall. Atherosclerosis is commonly known as a chronic inflammatory disease and is associated with certain risk factors such as hyperlipidemia, diabetes and hypertension. It is now widely accepted that ROS play an important role of the initiation and the development of atherosclerosis. Excess ROS production has been implicated in the pathogenesis of atherosclerosis and hypertension (Harrison, Griendling et al. 2003; Hamilton, Miller et al. 2004).

A widely accepted theory of atherogenesis postulates that it involves the formation of oxidized LDL by the actions of ROS (Steinberg 1987). The oxidized LDL binds to special LDL receptors, called scavenger receptors, expressed by macrophages and monocytes, resulting in uncontrolled LDL uptake and formation of foam cells. Binding with oxidized LDL initiates the activation of macrophages and monocytes and stimulates the expression of Mn-SOD. This process leads to the concentration of hydrogen peroxide increased by disturbing the steady-state levels of ROS (Dhalla, Temsah et al. 2000). Activated enzymes such as xanthine oxidase, NADPH oxidase or nitric oxide synthase can also produce more ROS which are associated with massive macrophage apoptosis and contributes thereby to the formation of atherosclerotic lesions (Sorg 2004).

To sum up, excess of fat, a defective metabolism of lipids, an infection, the presence of toxins or toxic metabolites or oxidative stress initiate the process and the oxidative stress promotes the development of the disease (Droge 2002).

1.5.5 Degenerative diseases

Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS) and Parkinson disease (PD) are neurogenerative disorders. These diseases are all have oxidative stress implicated in their pathogenesis (Foley and Riederer 2000; Manton, Volovik et al. 2004; Rosenstock, Carvalho et al. 2004).

Alzheimer's disease (AD) is a neurogenerative disorder characterized by a progressive decline in cognitive function and extensive neuronal loss. Its neuropathologic hallmarks are

neurofibrillary tangles and senile plaques, two forms of protein aggregation (Hardy and Selkoe 2002; Mattson 2002; Gsell, Jungkunz et al. 2004). The production of ROS in the brains of AD patients are implicated by the significant amount of lipid peroxidation detected in the brains as well as by the increased levels of 4-hydroxynonenal found in post-mortem cerebrospinal of AD patients (Montine, Kim et al. 1997). Furthermore, ROS were found to mediate protein damage of β-amyloid and hyperphosphorylated tau by aggregation (Multhaup, Ruppert et al. 1997). Oxidative damage to DNA, lipids and proteins haven been widely studied in AD. There was a significant increase of 8-OH-dG in nDNA and mtDNA isolated from cortical areas and from cerebellum of AD patients. Elevated levels of 8-OH-dG were detected in lymphocyte from AD patients. Lipid peroxidation and protein oxidation were found in AD brains via increased malondialdehyde (MDA) and protein carbonyl measurement (Mariani, Polidori et al. 2005).

Amyotrophic lateral sclerosis (ALS) is caused by the degeneration of motor neurons in the spinal cord or brain stem, resulting in skeletal muscle atrophy and weakness, and culminating in respiratory insufficiency. 10-15 % of ALS patients are inherited, among them 25-30 % are due to mutations of Cu/Zu SOD genes (Rosen, Siddique et al. 1993; Brown 1995). These abnormal forms of SOD are thus believed to play a role in the generation of an oxidative stress (Valentine, Hart et al. 1999). Increased levels of 8-OH-dG were found in plasma and urine of ALS patients which indicate oxidative damage to DNA. A large increase in protein carbonyls was found both in ALS frontal and motor cortex, indicating protein oxidation (Mariani, Polidori et al. 2005).

Parkinson's disease (PD) is the most common neurogenerative movement disorder. It is caused by the degeneration of dopaminergic neurons in *substantia nigra* (Olanow and Tatton 1999; Rao and Balachandran 2002). Autoxidation of dopamine and its catabolism by monoamine oxidase produce superoxide, hydrogen peroxide and hydroxyl radicals (Haavik, Almas et al. 1997). The metabolism of dopamine might be responsible for the high basal level of oxidative stress in *substantia nigra.* Thus, dopaminergic neurons are submitted to a chronic oxidative stress and can degenerate when ROS and free radicals are not efficiently neutralised by their antioxidant defences (Sorg 2004). PD was found to be associated with increased oxidative damage of DNA, shown by a marked enhancement in 8-OH-dG in caudatum and *substantia nigra*. Patients with PD showed an increase in chromosomal primary DNA damage and oxidative DNA damage in peripheral blood lymphocytes. There are also evidence of increased lipid peroxidation in the PD brain. A large increase in protein carbonyls was found in many PD brain regions (Mariani, Polidori et al. 2005).

1.5 Mechanism of oxidative damage to molecular targets in cells

Many ROS can react with almost all biological molecules, including DNA, RNA, lipids, carbohydrates, proteins, and antioxidants. The level of damage to particular targets depends on a number of factors which are: the concentration of target, rate constant for reaction of ROS with the target, the location of the target when compared with the site of ROS formation, the occurrence of chain reactions and damage transfer processes, and repair and scavenging reactions (Dean, Fu et al. 1997; Hawkins and Davies 2001; Davies 2005).

1.5.1 Oxidative damage to DNA

DNA is a key cellular component that is particularly susceptible to oxidative damage by ROS (Cerutti 1985). A number of alterations, such as, base modification, deoxyribose oxidation, strand breakage, and DNA-protein crosslinks, are due to reactions with ROS, especially hydroxyl radicals (Halliwell and Aruoma 1991; Mates, Perez-Gomez et al. 1999). Recent studies identified more than 100 different products of DNA damage by ROS (Dizdaroglu 1991; Cooke, Evans et al. 2003). DNA damage can result in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis (Marnett 2002; Cooke, Evans et al. 2003; Evans, Dizdaroglu et al. 2004).

Hydroxyl radical adds to the double bond of DNA-bases at diffusion-controlled rate. The second-order rate constant (k) of these reactions is in the range of $(3\text{-}10) \times 10^9$ $M^{-1}s^{-1}$ (Dizdaroglu, Jaruga et al. 2002). It abstracts an H-atom from the methyl group of thymine

and each of the five carbon atoms of 2'-deoxyribose at the rate constant of approximately

$2 \times 10^9 \, M^{-1}s^{-1}$ (Von Sonntag 1987). Addition reactions yield HO-adduct radicals of DNA

bases, whereas the allyl radical of thymine and carbon-centred sugar radicals are formed

from abstraction reactions. In the presence of oxygen, oxygen adds to HO-adduct radicals

and carbon-centred radicals at diffusion-controlled rates to give peroxyl radicals (Von

Sonntag 1987). Further reactions of base and sugar radicals generate a variety of modified

bases and sugars, base-free sites, strand breaks and DNA-protein cross-links (Valko,

Rhodes et al. 2006).

Hydroxyl radical adds to the C5- and C6- positions of thymine and cytosine, producing C5-

HO-and C6-HO-adduct radicals, respectively. The formation of cytosine glycol (Cg) and

thymine glycol (Tg) as a result of the oxidation of the C5-OH-adduct radicals of cytosine

and thymine, respectively, follows the addition of $HO^\bullet$. Oxygen adds to C5-HO-adduct

radicals at diffusion-controlled rates generating C5-HO-6-peroxyl radicals that may

eliminate $O_2^{\bullet-}$, followed by reaction with water to yield cytosine glycol and thymine glycol

(Dizdaroglu 1992; Breen and Murphy 1995). Oxidation of allyl radical of thymine

produces 5-hydroxylmetyluracil (5-HOMeUra). Addition of oxygen to the allyl radical

gives rise to 5-HOMeUra and 5-formyluracil (5-FoUra). Cytosine products are unique in

that they can deaminate and dehydrate. Thus, cytosine glycol yields 5-hydroycytosine (5-

HO-Cyt) by dehydration, uracil glycol by deamination, and 5-hydroyuracil (5-HO-Ura) by

deamination and dehydration (Dizdaroglu, Holwitt et al. 1986; Dizdaroglu, Laval et al.

1993).

Hydroxyl radical addes to purines giving rise to C4-HO-, C5-HO-, C8-HO adduct radicals. The most important lesion of DNA after ROS attacks, that is used extensively as a biomarker for cellular oxidative stress and genotoxicity in living organisms, is 8-hydroxy-2'-deoxyguanosine (8-HO-dG) or its oxidation product 8-oxo-2'-deoguanosine (8-oxo-dG) (Fig. 1.8) (Valavanidis, Vlahogianni et al. 2006). This oxidized DNA product is important because it is both relatively easily formed and is mutagenic and carcinogenic. It is a good biomarker of oxidative stress of an organism and a potential biomarker of carcinogenesis.
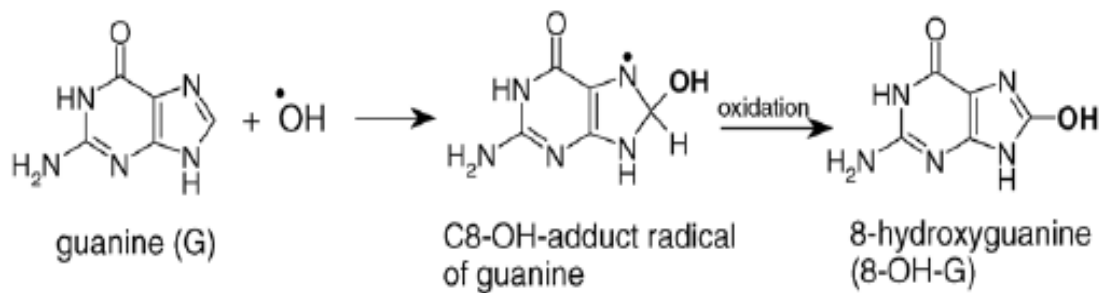
Figure 1.8   Reaction of guanine with hydroxyl radical

(From :Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M. and Mazur, M. (2006). "Free radicals, metals and antioxidants in oxidative stress-induced cancer." Chem Biol Interact **160**(1): page 9)

DNA-protein cross-link are also formed by free radical reactions (Oleinick, Chiu et al. 1987). A thymine-tyrosine cross-link was found in mammalian chromatin in vitro and in cells exposed to free radical generating systems (Margolis, Coxon et al. 1988). Different mechanism may involve the formation of DNA-protein cross-links (Dizdaroglu 1991). More recently, there is evidence that DNA-protein cross-links can caused by oxidized proteins, protein hydroperoxides (Gebicki and Gebicki 1999).

Mitochondrial DNA is generally considered more sensitive to oxidative damage than nuclear DNA. This result might be caused by mitochondrial DNA being the main target of ROS, because it lacks histone proteins to protect the DNA against oxidative damage, or because it has a limited DNA repair systems (Yakes and Van Houten 1997). The oxidative damage of mitochondrial DNA also involves base modification and strand breaks, which leads to formation of abnormal components of the electron transport chain. This results in the generation of more ROS by increasing leakage of electrons, and induces further cell damage. Eventually, oxidative damage to mitochondrial DNA may promote cancer and aging (Ames, Shigenaga et al. 1995; Bandyopadhyay, Das et al. 1999).

DNA as a target in vivo

Exposure cells to chemicals and ionizing radiation facilitate the formation of hydroxyl radicals which have potential to induce oxidative DNA damage. However, within the nucleus of eukaryotic cells, DNA is always combined with proteins. Chromatin DNA is in a complex with histones and many different nonhistone proteins. These proteins folding chromatin into a higher-order structure have been shown to protect DNA against strand breaking by hydroxyl radicals generated by ionizing radiation or Fenton reaction (Ljungman 1991; Enright, Miller et al. 1992; Xue, Friedman et al. 1994; Distel, Distel et al. 2002). Ljungman and his colleagues found out that the removal of DNA-bound proteins from the chromatin dramatically increased the frequency of radiation-induced DNA strand breaks (Ljungman 1991; Ljungman and Hanawalt 1992). After histones and nonhistone proteins were removed, DNA was 56-fold more radiosensitive than cellular DNA from Chinese hamster fibroblasts (Xue, Friedman et al. 1994). DNA in expanded chromatin

contained 14-fold fewer DNA strand breaks than naked, supercoiled DNA, whereas DNA in native and condensed chromatin contained about 100-fold and 300-fold fewer DNA strand breaks, respectively (Ljungman and Hanawalt 1992).

Exposure to gamma irradiation produces in mammalian cells DNA-protein crosslinks at approximately the same rate as double strand break (Xue, Friedman et al. 1994). Hydroxyl radical induced secondary protein radicals and protein peroxyl radicals cause DNA double strand break and DNA-protein crosslinks (Distel, Distel et al. 2002). Proteins bound to DNA protect DNA against radiation-induced damage. A study has shown that the binding of *lac* repressor to the wild-type *lac* operator protects DNA against the induction of strand breaks by ionizing radiation (Franchet-Beuzit, Spotheim-Maurizot et al. 1993). In 2001, Begusova and her colleagues elucidated that *lac* repressor proteins contribute to DNA conformational changes which protect DNA from radiation damage (Begusova, Eon et al. 2001). To sum up, the major primary target for ROS-induced oxidative stress is not DNA itself but its associated proteins.

1.5.2 Oxidative damage to lipids

Lipid peroxidation in cells is the one of the most widely investigated process induced by ROS, since cells have abundant membrane phospholipids at sites containing large amount of polyunsaturated fatty acid (PUFA) or PUFA side chain. The process of lipid peroxidation is a radical chain reaction (de Zwart, Meerman et al. 1999). PUFAs are very sensitive to oxidative reaction by ROS because of their carbon-carbon double bonds, which make it easy for ROS to abstract a hydrogen atom from an adjacent methylene carbon

group initiating lipid peroxidation (Reaction 1.27). The resulting carbon-centered radical may then undergo molecular rearrangement followed by interaction with oxygen to form a peroxyl radical (reaction 1.28).

I. Initiation of lipid peroxidation:

$$LH + R^\bullet \text{ or } HO^\bullet \rightarrow L^\bullet + RH \text{ or } H_2O \qquad (1.27)$$
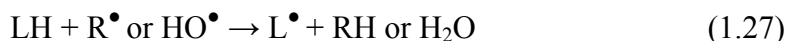
(LH= polyunsaturated lipid)

II. Propagation of lipid peroxidation:

$$L^\bullet + O_2 \rightarrow + LOO^\bullet \qquad (1.28)$$

($L^\bullet$= carbon-centered lipid radical)

($LOO^\bullet$ = lipid peroxyl radical)

$$LOO^\bullet + LH \rightarrow L^\bullet + LOOH \text{ (lipid hydroperoxide)} \qquad (1.29)$$

The peroxyl radicals are able the abstract a hydrogen atom from an adjacent PUFA side chain, propagating the chain reaction, and forming lipid hydroperoxides on the original PUFA (reaction 1.29) (Valavanidis, Vlahogianni et al. 2006).

Lipid peroxidation induces cell damage directly and indirectly. The direct effect is mainly effected by changing the membrane fluidity, so that peroxidized membranes become rigid and lose permeability and integrity (McIntosh, Advani et al. 1995). These results lead to unwanted substances cross the lipid bilayer, inactivate membrane- associated enzymes, and disturb receptor-involved cell signaling and nutrient uptake (Peck 1995).

The indirect effects of lipid peroxidation are the result of the formation of their breakdown products and intermediates. The lipid hydroperoxides can easily decompose into a variety

of breakdown products, especially in the presence of transition metals or metal complexes (Maiorino, Ursini et al. 1994). These products includes lipid alkoxyl radicals (LO$^\bullet$), aldehydes, alkanes, lipid epoxides, and alcohol (Griffiths, Moller et al. 2002). Most of these products are reported to possess cytotoxic, mutagenic and genotoxic properties (de Zwart, Meerman et al. 1999).

The major aldehyde products of lipid peroxidation are malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). The biological activities of MDA and HNE include cross-linking with DNA and proteins, which alter the function and activity of these molecules (Kautiainen, Vaca et al. 1992) and also have effect on receptors and signal transmission (Van der Vliet and Bast 1992). MDA appeared to be mutagenic in bacterial and mammalian cells and carcinogenic in rats (Marnett 1999). MDA reacts with nucleic acid bases to form multiple adducts, deoxyguanosine and deoxyadenosine. The mutagenic potential of MDA–DNA adducts (Marnett 1999) and nucleotide excision repair pathway has been found in *E. coli* (Fink, Reddy et al. 1997). Voitkun and Zhitkovich have found that malondialdehyde readily forms crosslinks between DNA and proteins (Voitkun and Zhitkovich 1999). MDA has been used as a convenient biomarker for lipid peroxidation. It can easily react with thiobarbituric acid (TBA) to form TBA-MDA adduct which is intensely colored chromogen measured by HPLC (Griffiths, Moller et al. 2002).

Hydroxynonenal is very reactive and the most cytotoxic aldehyde (Mario 2000). HNE is a major product of the oxidation of omega 6-polyunsaturated fatty acids, such as linoleic acid and arachidonic acid (Esterbauer, Benedetti et al. 1986; Siems, Grune et al. 1992). Because

HNE is not a substrate for aldehyde dehydrogenase, its half–life is quite long and it accumulates more readily than other aldehydes after oxidative stress. HNE is also a strong electrophilic agent that reacts preferentially with sulfhydryl (SH)-containing substances such as proteins (Morel, Tallineau et al. 1998). The Protein-bound HNE can lead to enzyme inhibition and cell injury. HNE can promote neuronal excitotoxicity by oxidative stress-induced impairment of glutamate transporter and other key metabolism enzymes in spinal cord injury patients (Siems, Grune et al. 1992; Springer, Azbill et al. 1997). Moreover, HNE was found in high doses in exudates from acute and chronic inflammations and so might play an important role as a chemical mediator of inflammation (Maggiora, Dianzani et al. 2002).

Consequently, the formation of lipid hydroperoxides and lipid hydroperoxide breakdown products are important biological events that can be used to monitor the extent of oxidative damage to lipids.

Lipids as target in vivo

Many experimental results had suggested that lipids are not critical initial molecular targets of ROS *in vivo*. Examples of such evidence include an important series of studies, showing that a significant increase in the polyunsaturated fatty acid content of mouse fibroblast cells had no effect in cell survival after X-irradiation (Wolters and Konings 1984; Goulet, Fisher et al. 1986). Damage observed with intact membranes of erythrocytes after irradiation are most likely the result of changes in the structure of membrane proteins rather than the lipids (Guille, Raison et al. 1987). This is also evidence that reactive oxygen species which initiate lipid peroxidation can cause direct damage to proteins which are associated with

membranes (Moosmann and Behl 2000), and protein oxidation may be more important in cell injury than the peroxidation of lipids (Caraceni, De Maria et al. 1997; Ciolino and Levine 1997).

A large number of studies demonstrating lipid oxidation under oxidative stress systems ranging from model membranes to living organisms can be resolved by the postulate that lipids are secondary rather than primary targets of ROS *in vivo,* with their oxidation triggered by reactions with activated primary target molecules and not directly with the ROS. Lipid oxidation is often not associated with ROS-initiated damage. For example, in neocortical synapses exposed to the ascorbate/Fe^{2+} system (Hensley, Carney et al. 1994), in absence of membrane breakdown in cardiomyocytes treated with radicals or oxidized lipids (Durot, Maupoil et al. 2000), and in hepatocytes injured by reperfusion, where cell damage was independent of lipid oxidation (Caraceni, Yao et al. 1994). Studies of the sequential oxidation of proteins, lipids and DNA in cultured cells also showed that lipids were secondary rather than primary targets of hydroxyl or peroxyl radicals (Gieseg, Duggan et al. 2000; Du and Gebicki 2002). Another experiment suggested that the lipid oxidation was a late event in oxidative damage in isolated rat liver mitochondria (Reinheckel, Noack et al. 1998). There are also reports demonstrating the ability of proteins to protect model liposome membranes from oxidation by hydroxyl and peroxyl free radicals (Tweeddale and Gebicki 2002). The protection of lipids by proteins from gamma irradiation, which can explain the lack of a role for lipids in ROS-induced cell death (Verma and Rastogi 1990). Liposome lipids were efficiently protected from HO$^{\bullet}$ radicals by external proteins, so that biological membranes were destroyed by removal of the associated proteins (Verma and

Rastogi 1990; Tweeddale and Gebicki 2002). To sum up, lipids are probably not primary targets of ROS-induced oxidative damage.

1.5.3 Oxidative damage to proteins

Exposure of proteins to ROS results in multiple changes in the target molecule. These include the oxidation of side-chain groups, backbone fragmentation (Davies and Delsignore 1987; Davies and Dean 1997), unfolding (Neuzil, Gebicki et al. 1993), crosslinking (Pacifici and Davies 1990), changes in hydrophobicity and conformation (Davies, Lin et al. 1987), altered susceptibility to proteolytic enzymes (Davies, Delsignore et al. 1987), and the formation of new reactive groups (e.g. hydroperoxides) (Gebicki and Gebicki 1993). Eventually, these processes can result in the loss of structural or enzymatic activity of the protein and, hence, biological perturbations (Lissi and Clavero 1990; Dean, Fu et al. 1997; Hawkins and Davies 2001). Many of these changes might be correlated to processes occurring in living organism resulting in further progress to ROS-related disease state (Davies 1987).

Free amino acids and amino acid residues in proteins are susceptible to oxidation by reactive oxygen species (ROS), such as hydroxyl radicals, peroxyl radicals and hydrogen peroxides. Radicals produced not only by ionizing radiation but also by Fenton reaction have been shown to oxidize amino acids and proteins (Stadtman 1993).

The mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides (Schuessler and Schilling 1984) and proteins

(Garrison, Jayko et al. 1962) were exposed to ionizing radiations under conditions where HO$^\bullet$ or a mixture of HO$^\bullet$/O$_2^{\bullet-}$ are formed (Stadtman and Levine 2003). The results of these studies demonstrated that reactions with hydroxyl radicals lead to abstraction of a hydrogen atom from the α-carbon of amino acids, the protein polypeptide backbone and also the aliphatic side chains of hydrophobic amino acid residues of proteins to form a carbon-centred radical, which under aerobic conditions reacts readily with O$_2$ to form peroxyl radicals. The peroxyl radicals are rapidly converted to the alkyl peroxides by reactions with the protonated form of superoxide radicals, (HO$_2$•), or by abstraction of a hydrogen atom from another molecule. Besides radiation, proteins are damaged by H$_2$O$_2$ only in the presence of in transition metals. Metal-catalyzed damage to proteins involves peptide bond cleavage (Stadtman 1990), oxidation of histidine residues (Davies 1987), bityrosine crosslinks (Davies, Delsignore et al. 1987), protein carbonylation (Davies and Delsignore 1987), and the formation of protein-centred alkyl, R•, alkoxyl, RO•, and alkylperoxyl, ROO•, radicals (Valko, Rhodes et al. 2006)

1.5.3.1 ROS induced damage on proteins-Peptide bond cleavage

Peptide bond cleavage occurs in protein oxidation by ROS. The protein alkoxyl radical and its derivatives are capable of undergoing peptide bond cleavage. Peptide bond cleavage can also occur by hydroxyl radical-initiated attack of the glutamyl and aspartyl residues of proteins to form a mixture of various products (Stadtman 1993). In addition, the oxidation of proline residues of proteins can induce peptide bond cleavage (Uchida, Kato et al. 1990; Dean, Fu et al. 1997).

1.5.3.2 Oxidation of amino acid residues in proteins

Aliphatic amino acid oxidation

The radical-mediated reaction in aliphatic residues occurs mainly by hydrogen atom abstraction processes that give rise to carbon-centred radicals (Davies 2005). Dimersation with another radical is most likely to be a common reaction in the absence of oxygen, whereas in the presence of oxygen, peroxyl radicals are produced. These peroxyl radicals undergo further reactions and hence can give rise to hydroperoxides via hydrogen abstraction from C-H bonds, hydroxides and carbonyl groups (Davies and Dean 1997). Recent studies have suggested that hydroperoxides can be the major products of some free amino acids when exposed to ROS in the presence of oxygen (Simpson, Narita et al. 1992; Gebicki and Gebicki 1993). These hydroperoxides undergo an additional reaction, such as one-electron reduction, and give rise to the formation of further radicals, such as alkoxyl, carbon-centred and superoxide radicals and also alcohols and carbonyl compounds (Davies, Fu et al. 1995). Many studies have been indicated these hydroperoxides can induce oxidation of a wide range of other biological targets including DNA (Gebicki and Gebicki 1999; Luxford, Morin et al. 1999; Luxford, Dean et al. 2000; Luxford, Dean et al. 2002), lipids and other proteins (Hampton, Morgan et al. 2002; Morgan, Dean et al. 2004).

Cysteine and methionine oxidation

Methionine and cysteine have reactive sulfur-containing side chains that represent major target of ROS (Levine, Mosoni et al. 1996). Oxidation of methionine and cysteine residues in proteins is reversible.

Reaction of cysteine with hydroxyl radicals occurs very rapidly and give rise to thiyl radicals (RS•) by hydrogen abstraction. In the present of oxygen, these radicals react rapidly with oxygen to form a peroxyl species, $RSOO^{\bullet}$ and a peroxide, RSOOH (Von Sonntag 1987). The thiyl radical can react with another thiol anion, to give a disulfide radical anion. Therefore, at physiological pH, the formation of disulfide bonds may be the most likely consequence of cysteine oxidation (Davies and Dean 1997). The disulfides can be reduced back to thiols by glutathione or other thiols (Hoshi and Heinemann 2001; Jones, Go et al. 2004).

Methionine is oxidized to methionine sulfoxide by many different ROS, such as H_2O_2, hydroxyl radicals, hypochlorite, chloramines, and peroxynitrite, all these oxidants being produced in biological systems (Vogt 1995). Methionine sulfoxide can be reduced back to methionine by methionine sulfoxide reductase. The oxidation of surface-exposed methionine residues in proteins can thus protect other essential amino acid residues from oxidative damage (Levine, Mosoni et al. 1996; Hoshi and Heinemann 2001).

Aromatic amino acid oxidation

The aromatic amino acid residues of protein are major targets for ROS-induced oxidation. Phenylalanine residues are oxidized by hydroxyl radicals to form *ortho-*, *para-* and *meta-*tyrosine derivatives (Maskos, Rush et al. 1992). A major product of hydroxy-radical addition to tyrosine is 3,4-dihydroxyphenylalanine (DOPA) which has reducing properties (Gieseg, Simpson et al. 1993). Protein-bound DOPA (PB-DOPA) has been shown to be a major component of the stable reducing species formed during protein oxidation under

several conditions. Either DOPA or PB-DOPA can mediate oxidative damage to DNA via the formation of 8-oxodG and 5-OHdC. Thus, PB-DOPA was able to promote further radical-generating events, which then transferred damage to other biomolecules such as DNA (Davies and Dean 1997; Morin, Davies et al. 1998; Hawkins and Davies 2001).

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Reaction with a number of more selective oxidant (e.g. $N_3^\bullet$) with tyrosine residues very rapidly and efficiently gives rise to tyrosine phenoxyl radicals. These radicals undergo dimerisation and produce the cross-linked product, di-tyrosine (Heinecke, Li et al. 1993). This can induce the formation of intra- or inter molecular cross-links in proteins. In the presence of O_2, hydroperoxide species are generated by at addition of superoxide radicals on the tyrosine phenoxyl radicals (Davies and Dean 1997).

Rapid $HO^\bullet$ addition to the aromatic rings also happens in trytophan and histidine residues in proteins. In the case of Trp, $HO^\bullet$ is added to the benzene ring to produce 5- and 7-hydroxytryptophan in the absence of oxygen. However, the $TrpOO^\bullet$ is generated in the presence of oxygen. The peroxyl radicals as the products of a result of initial reaction of oxygen and $HO^\bullet$ can give rise to N-formylkynurenine and kynureine (Maskos, Rush et al. 1992).

Histidine can be oxidized by ROS in the aromatic amino acid-like oxidative mechanism. $HO^\bullet$ radicals add to the imidazole ring of histidine and form the oxidation products including asparagines, aspartic acid (Davies and Dean 1997) and 2-oxo-histidine (Uchida and Kawakishi 1993; Lewisch and Levine 1999). Histidine is often associated with

metalloenzymes, so that oxidation of histidine resides can cause of inactivation of enzymes (Zhao, Ghezzo-Schoneich et al. 1997). The 2-oxo-histidine is generated as the main product in the oxidative modified proteins by ROS. Therefore, the formation of 2-oxo-histidine has been reported as a general marker of protein oxidation (Uchida and Kawakishi 1993; Davies, Fu et al. 1999).

1.5.3.3 ROS induced damage on proteins-protein cross linkage

Protein oxidation by ROS can give rise to inter- and intra-protein cross-linkages by different mechanisms, including: (1) interaction of two carbon-centred radicals obtained by the hydroxyl radicals-driven abstraction of hydrogen from the polypeptide backbone (Garrison 1987); (2) the oxidation of tyrosine to from bityrosine cross-links; (3) the oxidation of sulphydryl groups of cysteine residues to form disulfide cross-links (Enescu and Cardey 2006); (4) the addition of lysine amino groups to the carbonyl group of an oxidized protein; (5) the reactions of both aldehyde groups with two lysine residues in the same or two different protein molecules; (6) the interaction of glycation/glycoxidation derived protein carbonyls with either a lysine or an arginine residue of in the same or two different protein molecules (Wells-Knecht, Zyzak et al. 1995).

1.5.3.4 Site-specific metal-catalyzed oxidation

The side chain of amino acid residues of proteins is subject to oxidation by metal-catalyzed oxidation (MCO) systems (Stadtman 1990). The side-chains of lysine, arginine, proline, histidine, cysteine, and threonine residues in protein are highly sensitive to oxidation by redox metals, this process is site-specific. It is believed that Fe (II) binds to both high- and

low-affinity metal-binding site on the protein. This Fe (II)-protein complex reacts with H_2O_2 via Fenton reaction to yield hydroxyl radicals at the site. These hydroxyl radicals then induce further oxidative damage to the amino acid residues (Stadtman 1993; Valko, Rhodes et al. 2006). The Fe (II) reacts with other amino acid residues leading to the generation of carbonyl derivatives. In the studies with bacterial glutamine synthetase, it was indicated that amino acid residues located at metal binding sites on the enzyme are very sensitive to metal-catalyzed oxidation. Oxidation leads to the appearance of carbonyl groups in amino acid side chains of the protein (Climent and Levine 1991).

In addition, PB-DOPA can bind and redox cycle transition metals, thereby potentially controlling and/or localising further oxidation of proteins and other macromolecules (Morin, Davies et al. 1998). The redox activity of PB-DOPA can result in chemical modifications to other macromolecules, and although these are often damaging, and prevent normal function, this implies that chemical signalling via PB-DOPA might occur. On the other hand, PB-DOPA does bind transition transition metals very firmly, so that in the absence of appropriate co-reactants, this may help to sequester them away from oxidative participation. PB-DOPA is thus both redox active, and a potential player in oxidative events; a potential antioxidant by virtue of its metal binding capacity. This antioxidant activity is analogous to that of some chemical chelators, which may limit or promote the redox activity of bound metals depending on their specific chemistry (Nelson, Foxwell et al. 2007).

1.5.3.5 Cellular metabolism of oxidized proteins-enzymic removal

There are two major pathways for oxidized proteins degradation in mammalian cells: lysosomal proteases and the proteasome complex. Oxidized proteins are sensitive for proteolytic digestion (Davies, Lin et al. 1987).

Proteasomes are multicatalytic proteinases present in a wide variety of cells (Dean, Fu et al. 1997). They are mainly found in the cytosol, but are also present in the nuclei of mammalian cells. In the nuclei, proteasomes are soluble or loosely attached to the chromatin. In the microsomes, proteasomes are on the outside of the membranes (Palmer, Rivett et al. 1996). Due to the oxidative rearrangement of secondary and tertiary protein structure, there is an increase of surface hydrophobicity of all oxidatively modified proteins. Thus, the proteasome complex can recognize hydrophobic residues and catalyze selective removal of the modified cellular proteins. Even thought the nuclear function of proteasomes is unclear, it is possible that oxidized nuclear proteins such as histones are their potential target. The 20S proteasome can remove oxidized histones and histone-DNA complexex *in vitro*. However, excessive oxidative damage to proteins inhibits their proteolytic susceptibility (Ullrich, Sitte et al. 1999). In the year 2000, Sitte and his colleagues found that an accumulation of oxidized proteins such as lipofuscin/ceroid may actually cause further increases in damage accumulation during aging by inhibiting the proteasome (Sitte, Huber et al. 2000). Proteasome plays an essential role of minimizing the oxidative protein-induced damage by limiting protein aggregation and cross-linking and by removing potentially toxic protein fragments (Grune, Reinheckel et al. 1997).

A water-soluble protease which is capable of cleaving proteins at or near a membrane/aqueous interface had been studied in the degradation of membrane bound oxidized proteins in erythrocytes (Beppu, Inoue et al. 1994). A membrane-bound or integrated protease in ER lumen can regulate ER protein degradation (Young, Kane et al. 1993). Within cells there are many potential proteolytic sites, such as mitochondria, lysosomes and proteasomes, for degradation of oxidized local or transported proteins (Dean, Fu et al. 1997). Mitochondria contains a proteolytic system which is able to cleave oxidatively denatured proteins under alkaline conditions (pH 8.0) in an ATP-independent pathway (Marcillat, Zhang et al. 1988). Lysosomes, intracellular compartments containing a range of hydrolytic enzymes which selectively recognize and degrade oxidatively damaged proteins and peptides, play an important role in cellular processes (Szweda, Friguet et al. 2002).

Oxidized proteins that enter cells via endocytosis, pinocytosis, or phagocytosis can also be degraded by lysosomes. Degradation of extracellular oxidized protein by lysosomes may depend on the presence of specific receptors on the plasma membrane and the degree of oxidative modification of the substrate (Jessup, Mander et al. 1992; Grant, Jessup et al. 1993). For example, the accumulation of oxidized LDL within macrophage is due to the formation of inter-or intra-molecular crosslinks in apoB which turn into molecules less available to proteolysis (Jessup, Mander et al. 1992; Dean, Fu et al. 1997).

These proteolytic pathways of oxidized proteins can provide a valuable line of secondary antioxidant defense in cells.

1.5.3.6 The biological damage by oxidized proteins

Oxidative damage of proteins is an important event *in vivo*, considering the numerous proteins involved in receptors, enzymes, single proteins, structural proteins, transport proteins, and so on. It is well-established that oxidation of protein can induce a wide range of biological damage. Oxidation of protein side-chain can give rise to unfolding and conformational changes in proteins and this can have consequential effects on biological functions. Oxidation of both backbone and the side chains can results in the formation of further reactive species. These include the formation of hydroperoxides or peroxides, DOPA and other short-lived intermediates. It has been suggested that reaction of peroxides with reducing metal ions, or UV light can give rise further radicals. These radicals can also induce damage to other biomolecules and generate further damage (Davies 2005).

1.6 Proteins are the primary target of ROS damage

1.6.1 Stepwise development of ROS-induced biological damage

The processes implicated in ROS-mediated pathology proceed in several stages (Gebicki, Du et al. 2000) (Fig. 1.9).

1. Oxidative stress

2. Primary ROS: $O_2^{\bullet-}$
Secondary ROS:
H_2O_2 (Enzyme-mediated)
$HO^{\bullet}$ (Fenton reaction)

3. Primary target molecules
Proteins

6. Pathology or death

4. Secondary target molecules
Lipid
DNA
Carbohydrates
Antioxidants

5. Cell injury
Tissue damage
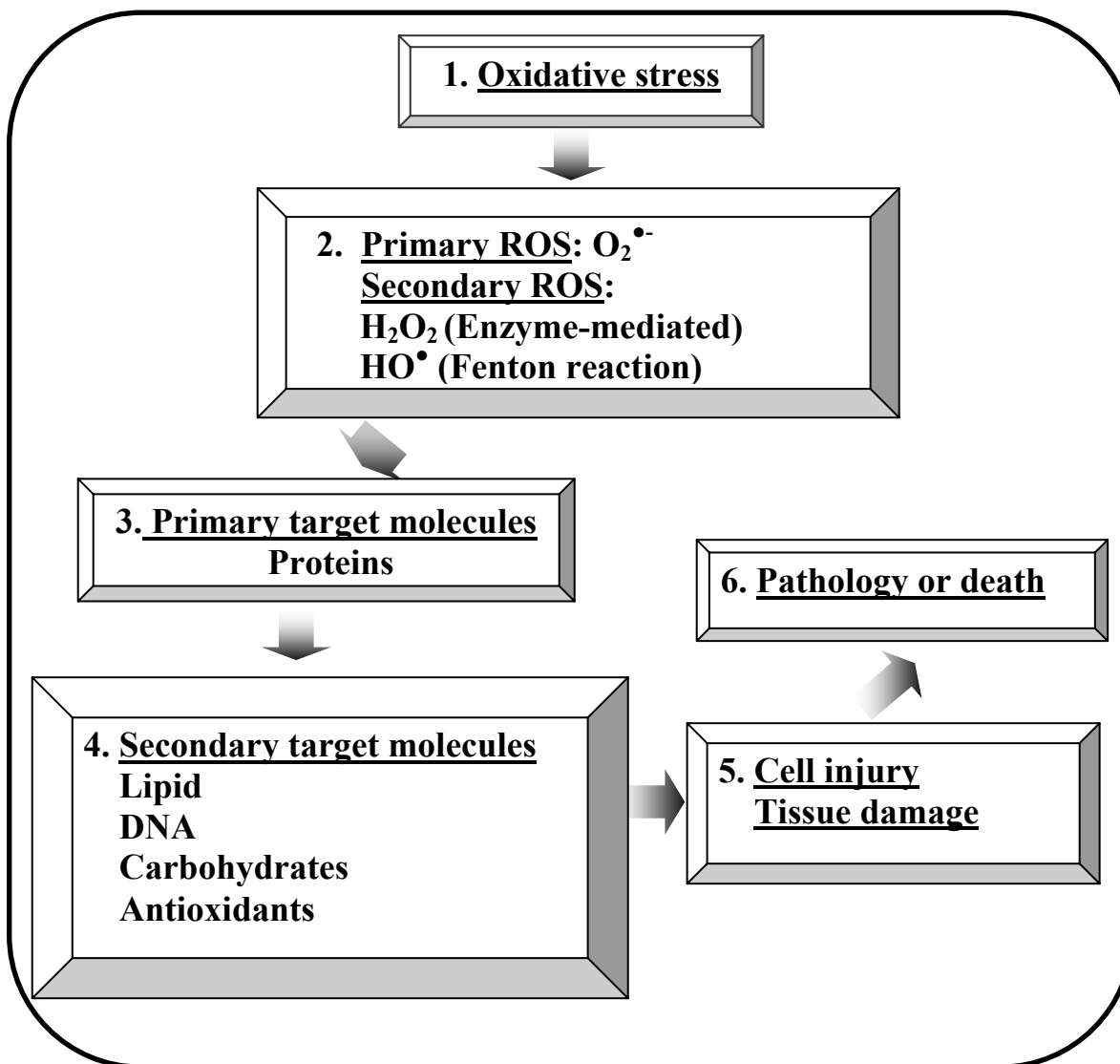
Figure 1.9 The reactive oxygen species-mediated pathological process

Stage 1: oxidative stress; stage 2: ROS (primary and secondary ROS); stage 3: Primary target molecules; Stage 4: secondary target molecules; stage 5: general cell/tissue damage; stage 6: pathology and death.

The first stage occurs as the result of chronic or periodic oxidative stress. Superoxide radicals ($O_2^{\bullet-}$), the earliest formed and the most abundant radicals in aerobic cells, are the most significant general primary ROS. While chemically only weakly reactive, superoxide is believed to be the principal initiator of biological damage by its ability to generate powerful oxidants under physiologically possible conditions. These relatively unreactive radicals act as a precursor of secondary ROS (Gebicki, Du et al. 2000). They react spontaneously with themselves or via enzyme-catalysis yielding hydrogen peroxide (H_2O_2) (Pryor 1986; Martinez-Cayuela 1995; Dreher and Junod 1996). Hydrogen peroxide is then involved in a metal-catalyzed Fenton reaction, which can produce hydroxyl radicals.

Furthermore, superoxide can act as an additional source of the peroxide and reductant of the metal, with the final formation of the radical considered the most damaging *in vivo*, the hydroxyl ($HO^{\bullet}$). Hydroxyl radical ($HO^{\bullet}$) is extremely reactive, rapidly reacts with all biological molecules in a diffusion-controlled rate (Table 1.5) (Buxton, Greenstock et al. 1988; Davies 2005) and forms other radicals (especially peroxyl radicals) in cells (Dreher and Junod 1996; Gebicki 1997). Control at the $HO^{\bullet}$ level cannot be achieved, because of their inevitable reactions with cell components, and which cannot be scavenged by antioxidants (Von Sonntag and Schuchmann 1994).

Table 1.5 Rate constant for reactions of HO$^\bullet$ with macromolecules at pH 7.0

Substrate	Rate constant ($dm^3\,mol^{-1}\,s^{-1}$)
DNA	8×10^8
RNA	1×10^9
Linoleic acid	9×10^9
Albumin	8×10^{10}

(Selected data from Davies, M. J. (2005). "The oxidative environment and protein damage." Biochim Biophys Acta **1703**(2): page 94.)

1.6.2 Proteins as primary target

Many reactive oxygen species can attack vital cell components like nucleic acid, polyunsaturated fatty acids, and proteins. It is generally accepted that these crucial target molecules are DNA, lipids and proteins (Pryor 1986). Whether one of them is the predominant target and, if so which one, are subject of much discussion, whose outcome is important for future research because benign interruption of the steps linking oxidative stress with pathology would prevent or reduce the resultant damage (Gebicki 1997).

Since the protection of primary target molecules from ROS attack gives the best chance of control of reactive oxygen species-mediated pathological processes, these molecules should be identified. They are now defined as proteins, not DNA or lipid (Caraceni, De Maria et al. 1997; Gieseg, Duggan et al. 2000; Brookes, Levonen et al. 2002). Direct evidence that proteins are such targets in biological systems is provided by many recent studies.

Hydroxyl radicals are generally believed to be the most important direct agents of ROS-induced biological damage. However, the determination of their formation and concentrations *in vivo* is difficult because of their high reactivity, short diffusion path and the impossibility of scavenging with achievable concentrations of reagents such as mannitol or DMSO. Based on its high oxidation potential, randomly generated $HO^{\bullet}$ attack individual components in a mixture of molecules largely according to their relative masses. Proteins, which contribute 75 % of dry cell mass, comprise the major, non-water, components in liver, leukocyte and plasma (Gebicki 1997).

While the lifetime of hydroxyl radicals is only about 10^{-9} seconds (Sies 1991), the radicals react within only about 6 nm from their point of formation (Roots and Okada 1975). When hydroxyl radicals are generated randomly, such as in cells exposed to ionizing radiation, their high reactivity and short diffusion distance imply that the most abundant cell component (excluding water) is the most probable to be attacked. Based on this kinetic data, proteins, the most abundant component in cells, are the major sites of ROS-induced damage (Gebicki 1997). The data given in Table 1.5 shows that the rate constants for the reaction of hydroxyl radicals with biomolecules differ in small degree, and therefore, the overall rate of reaction will be driven by the concentration of the target (Davies 2005).Thus, the low probability of direct attack by ROS on lipids and DNA in biological systems makes proteins as the most likely initial target molecules (Pryor 1986).
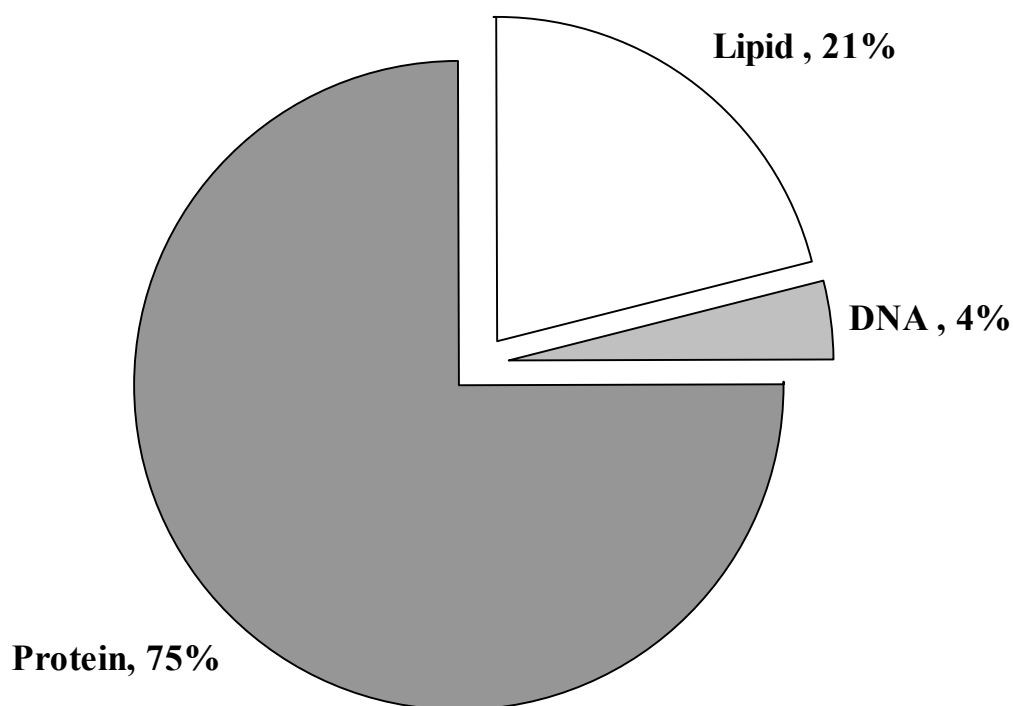
Figure 1.10 Relative amounts of protein, DNA and lipid in typical eukaryotic cells The calculations are based on from data reference (Gebicki 1997).

For a long time, DNA and lipids always were considered main vital targets for ROS. DNA is now believed to be a secondary rather than a primary target of ROS. For example, DNA was not the initial site of damage by ROS in hepatocytes (Caraceni, De Maria et al. 1997) and mouse myeloma cells (Brookes, Levonen et al. 2002). Histone proteins exposed to hydroxyl radicals, generated by γ-radiation, in the presence of oxygen acquire protein-bound hydroperoxides in a dose dependant manner. These histone hydroperoxides are relatively stable, however, in the presence of copper ions decompose rapidly to give a variety of radicals which can react both pymidine and purine nucleobases (Luxford, Dean

et al. 2000). Thus the radical-induced nuclear protein damage can give rise to DNA damage, including both DNA-protein crosslinks and the formation of oxidized DNA bases (Simandan, Sun et al. 1998; Luxford, Dean et al. 2002). There is strong evidence that early and random damage to proteins was produced before lipid peroxidation in rat liver mitochondria (Reinheckel, Noack et al. 1998), injured hepatocytes (Garrison, Jayko et al. 1962) and U937 cell cultures (Gieseg, Duggan et al. 2000). Gebicki and his colleagues showed that lipid membranes are likely to be effectively protected from randomly-generated hydroxyl radicals by proteins. Thus, lipid peroxidation can be inhibited by proteins through the preferential scavenging of the radicals. This generates protein radicals which are unable to oxidize membrane lipids. Again, protein peroxyl radicals and hydroperoxides constitute an important hazard to biological systems under oxidative stress (Tweeddale and Gebicki 2002).

1.7 Protein hydroperoxides

While ROS can cause oxidative damage to proteins *in vivo,* for many years proteins were not considered to be significant targets of ROS (Halliwell 1988), because there was no evidence of damage by the altered proteins to other cell components. This was provided by the discovery of formation of reactive hydroperoxide groups in proteins attacked by biologically significant ROS (Simpson, Narita et al. 1992). Proteins attacked by most ROS result in formation of protein carbon-centred free radicals which can be rapidly converted to peroxyl radicals in the presence of oxygen (Simpson, Narita et al. 1992). Long-lived protein hydroperoxides are then generated. Subsequent work showed that the altered

proteins can damage DNA, enzymes (Du and Gebicki 2004), antioxidants, lipids, generate further reactive free radicals and modulate apoptosis. It is now clear that oxidized proteins are a new form of ROS (Gebicki 1997). Lipids, DNA, carbohydrates and other proteins then act as secondary molecules in this ROS-induced pathology process (Simpson, Narita et al. 1992; Gebicki and Gebicki 1993; Gebicki 1997; Simandan, Sun et al. 1998; Luxford, Morin et al. 1999). Yet, protein hydroperoxides are a major product of low density lipoprotein oxidation during copper, peroxyl radical and macrophage-mediated oxidation (Gieseg, Pearson et al. 2003) .

1.7.1 The history of amino acid and protein hydroperoxides studies

There is a multitude of different studies of protein oxidation. Among them, the hydroperoxides, the reactive non-radical products of protein oxidation, are of main interest in my research.

In 1942, the formation of amino acid and protein hydroperoxides was first published by Latarjet and Loiseleur. They exposed a range of compounds in aqueous solution to X-rays and tested for the formation of peroxides by an iodometric technique. The results showed that: horse serum albumin, glycine, alanine, leucine, aspartic acid and lysine gave positive results, but ovalbumin was inert. After almost 20 years, the first systematic study of the formation of hydroperoxides in a range of amino acids and short peptides by X-ray in air-saturated unbuffered aqueous solutions was performed in 1958. Okada obtained results showing that isoleucine, leucine, norleucine, glutamate, lysine, arginine, di- and tri-peptides produced high peroxide yields, while glycine and alanine did not. Three years later, Ambe

and Tappel irradiated cytochrome c and hemoglobin by γ-rays. Two tests were used to detect oxidized products: an iodometric assay for peroxides and the thiobarbituric acid (TBA) reaction for lipid peroxidation. There was no formation of protein peroxides in TBA reaction assay. In 1962, the protein hydroperoxides production was again reported (Garrison, Jayko et al. 1962).

The specificity of aerobic iodometric peroxide assay was improved by the destruction of H_2O_2 with catalase. Based on this approach, the generation of BSA (Bovine Serum Albumin) and serum protein peroxides under γ-irradiation was detected (Gebicki and Guille 1988). Recent studies showed that protein damage caused by hydroxyl radicals leads in the presence of oxygen to the formation of an intermediate peroxyl radical which were subsequently reduced to long-lived hydroperoxides. 10-50 % of all antioxidant potential of human plasma challenged with peroxyl radicals was due to proteins. This suggested that protein-free radical interaction may be common and may have considerable significance *in vivo* (Simpson, Narita et al. 1992; Gebicki and Gebicki 1993). Under Fenton reaction, hydroxyl radicals transfer from oxidized myoglobins (Mb) to other proteins. Fe^{3+} Mb reacted with hydrogen peroxide yields Fe^{5+}-oxo species at heme centre and protein (globin)-derived radicals at tyrosine (Tyr-103) and tryptophan (Trp-14). Tryptophan-derived peroxyl radicals can rapidly react with a wide range of proteins to give rise long-lived secondary radicals (Irwin, Ostdal et al. 1999). Three methods were used in peroxidation of BSA. The hydroxyl radicals were formed by γ-irradiation and Fenton reaction, while peroxyl radicals were generated via the thermal decomposition of bis-azo compound 2, 2'-azobis-(2-amidinopropane) dihydrochloride (AAPH). BSA hydroperoxides

(BSA-OOH) were then generated and remained at 30 % of original level after 1-week storage (Simpson, Narita et al. 1992; Ostdal, Davies et al. 2002).

1.7.2 Formation and properties of protein hydroperoxides

The main free radical responsible for protein peroxidation was defined as hydroxyl radicals. The study for the formation of hydroperoxides from proteins exposed to a range of oxidants showed that both Fenton reaction and ionizing radiation, the major hydroxyl radical generation systems, could produce high level of oxidative products (Gebicki 1997); while other ROS, such as $O_2^{\bullet-}$ and H_2O_2, were ineffective.

The mechanism for the formation of protein hydroperoxides by hydroxyl radicals is shown below.

$$PrH + HO^{\bullet} \rightarrow Pr^{\bullet} + H_2O \qquad (1.30)$$

$$Pr^{\bullet} + O_2 \rightarrow PrOO^{\bullet} \qquad (1.31)$$

$$PrOO^{\bullet} + e^- \rightarrow PrOO^- \qquad (1.32)$$

$$PrOO^- + H^+ \rightarrow PrOOH \qquad (1.33)$$

$$PrOO^{\bullet} + PrH \rightarrow PrOOH + Pr^{\bullet} \qquad (1.34)$$
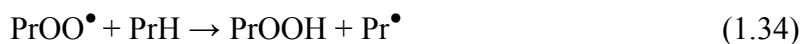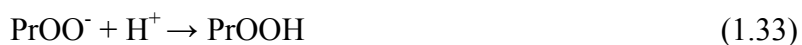
PrH is the protein molecule, with H the atom abstracted in the initial reaction with hydroxyl radical (reaction 1.30). $Pr^{\bullet}$ is a carbon-centred radical with lifetime long enough for reaction with oxygen before decomposing (reaction 1.31). The free electrons translocation in proteins could reduce protein peroxyl radical in reaction (1.32); electrons might come from as $O_2^{\bullet-}$ shown in previous study. Direct hydrogen abstraction probably occurs from

other parts of the protein molecule by PrOO$^\bullet$ (reaction 1.34), then a short chain reaction initiates (Neuzil, Gebicki et al. 1993). This could be terminated when protein free radical in the chain react with O_2 to form a hydroperoxide or decomposes. Eventually, protein hydroperoxides are generated (Gebicki and Gebicki 1993; Gebicki 1997; Gebicki, Du et al. 2000; Hawkins and Davies 2001).

1.7.3 Biological significance of protein hydroperoxides

Protein hydroperoxides are generated in high yields at both side-chain and α-carbon backbone (Davies, Fu et al. 1995; Davies 1996; Morin, Davies et al. 1998; Davies, Fu et al. 1999; Headlam and Davies 2003; Wright, Hawkins et al. 2003). Hydroperoxides can also appear on amino acids. There are six amino acids known to produce significant amount of amino acid peroxide: glutamate, lysine, proline, valine, leucine, and isoleucine. All of these amino acids have a tertiary carbon or at least two methylene groups, which provide stability to the amino acid radicals to form hydroperoxides (Gebicki and Gebicki 1993; Gebicki 1997). The potential biological importance of protein/amino acid hydroperoxides relies on their stability and reactivity. Once formed, the long lifetimes allow the protein/amino acid peroxides to diffuse through intra and extra cellular spaces, and then initiate further chemical reactions (Gebicki 1997; Du and Gebicki 2004).

Protein hydroperoxides can damage DNA, lipids, enzymes and antioxidants and generate a range of further reactive free radicals (Fig. 1.11). Protein/amino acid hydroperoxides can give rise to reactive free radicals. They can be catalytically degraded by transition metal ions, such as iron and copper (reaction 1.35 and 1.36) (Davies, Fu et al. 1995).

$$PrOOH + Fe^{2+} \rightarrow PrO^{\bullet} + Fe^{3+} + HO^{-} \qquad (1.35)$$

$$PrOOH + Fe^{3+} \rightarrow PrOO^{\bullet} + Fe^{2+} + H^{+} \qquad (1.36)$$
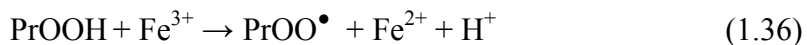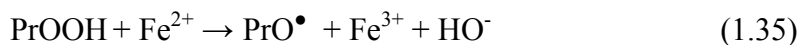
The radicals produced from these reactions are alkoxyl and peroxyl radicals which can initiate further damage to either the parent proteins or other molecules (the formation of organic and lipid peroxides) and carry on new reactions (Davies 1996; Gebicki 1997). Exposure to gamma-irradiation of N-acetyl amino acids and peptides also gives high levels of hydroperoxides in the presence of oxygen. Decomposition of alpha-carbon hydroperoxides by Fe^{2+}-EDTA leads to the formation of alkoxyl radicals and releases a range of products, including $CO_2^{\bullet-}$, carbon centred specie, and radical intermediates of $^{\bullet}C$ (O) NHR radicals. Subsequently, fragmentation of the proteins occurs. These reactions may be propagating processes during protein chain oxidation (Davies 1996).

Previous studies have shown that protein hydroperoxides can be reduced by a variety of reducing agents, such as ascorbate, glutathione, glutathione peroxidase and chemical agents, $NaBH_4$, triphenylphosphate, and dithionite (Simpson, Narita et al. 1992; Gebicki and Gebicki 1993). In addition, human plasma and mouse macrophages have been reported to have the ability to reduce protein hydroperoxides (Fu, Hick et al. 1995). Amino acid hydroperoxides can be completely removed by most of the reductants, however, protein hydroperoxides can only removed partially by reducing agents. This result might due to the steric hindrance of protein hydroperoxides (Davies, Fu et al. 1995). Overall, the hydroxides derived from the amino acid/ protein hydroperoxides provide a useful stable *in vivo* marker for studying protein oxidation under oxidative stress (Fu, Gebicki et al. 1995).
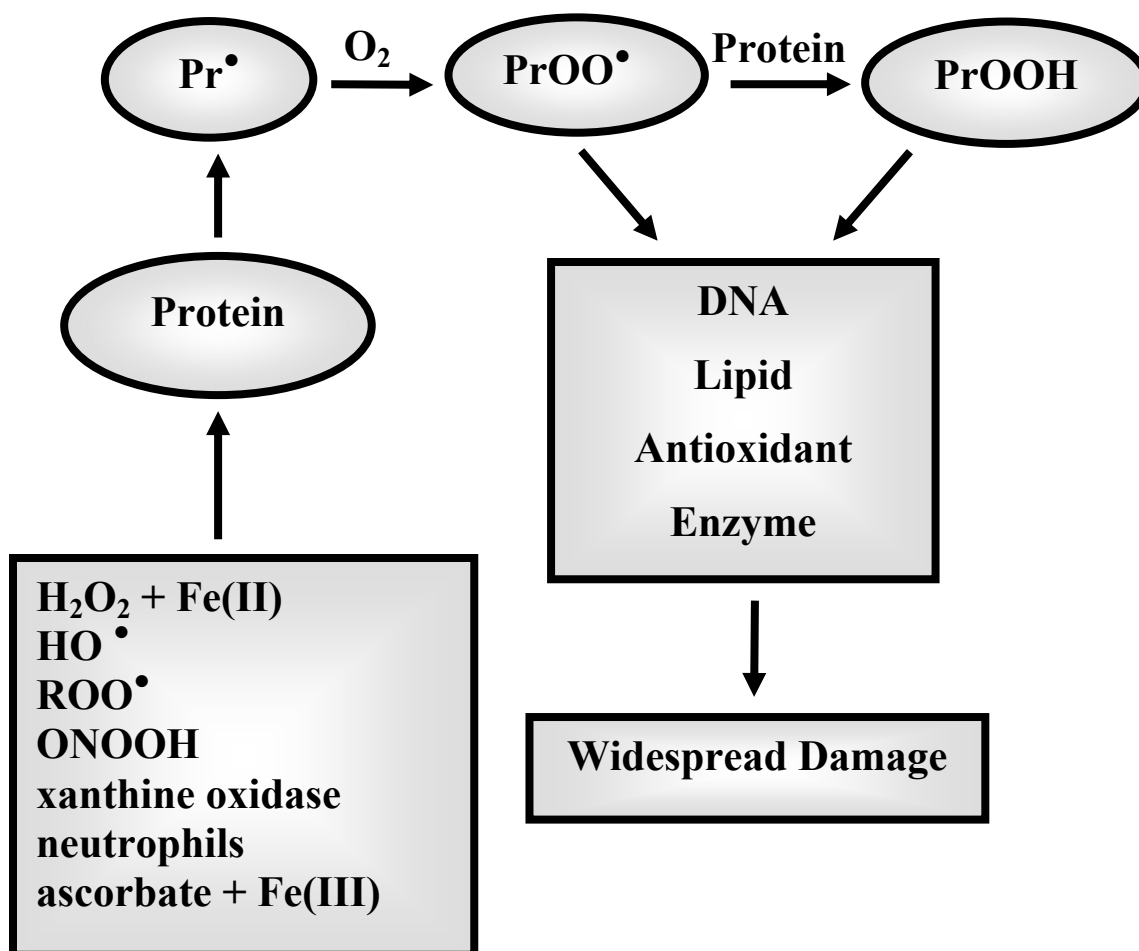
Figure 1.11 Proposed roles of proteins in the transmission of biological damage initiated by reactive oxygen species

(The figure is modified from: Gebicki, J. M., Du, J., Collins, J. and Tweeddale, H. (2000) Acta Biochimica Polonica 47, page: 908.)

In contrast, hydroperoxides can also consume important cellular antioxidants (low molecular weight) via redox reaction and increased oxidative stress (Ostdal, Davies et al. 2002). The reduction of protein hydroperoxides by ascorbate and glutathione is relatively slow compared with the reaction with Fe^{2+} salt or Fe^{2+}-DETA (Simpson, Narita et al. 1992). Glutathione and ascorbate *in vivo* could be oxidized by protein peroxides, resulting in loss of resistance to ROS (Simpson, Narita et al. 1992; Gebicki and Gebicki 1993; Headlam and Davies 2003). In the case of GSH/GSH peroxidase, GSH itself was consumed by hydroperoxide groups and glutathione peroxidase has shown an obvious catalytic effect on degradation of hydroperoxide groups on small proteins (Gebicki, Gill et al. 2002). In addition, protein peroxides have the potential for selective inactivation of enzymes, such glutathione peroxidase (Gebicki 1997; Du and Gebicki 2002), glyceraldehydes-3-3 phosphate dehydrogenase (Morgan, Dean et al. 2002), and cellular caspases (Hampton, Morgan et al. 2002). Peroxidized amino acids (such valine, leucine, and lysine) and peroxidized proteins (insulin and lysozyme) have the ability to inactivate glutathione reductase (Gebicki 1997; Morgan, Dean et al. 2002) .

Formation of DNA-protein crosslinks induced by protein hydroperoxides strongly depended on the intact hydroperoxide groups on the proteins. Incubation of Cu^+ and other transition metal with peroxidized individual DNA proteins (H1, H2A, H2B, H3, or H4) and histone octamers induced radicals which reacted readily with pyrimidine DNA bases and nucleosides, and gave protein-DNA base crosslinks. Radicals from histone H1-hydroperoxides, and other protein and amino acid hydroperoxides can also oxidize both free 2'-deoxyguanosine and intact calf thymus DNA to give the oxidized base 7, 8-dihydro-

8-oxo-2'-deoxyguanosine (8-hydroxy-2'-deoxyguanosine, 8-oxodG) (Luxford, Morin et al. 1999). With uridine the histone hydroperoxide-derived radicals undergo addition across the C_5-C_6 double bond of the pyrimidine ring to give cross-linked adduct species (Luxford, Dean et al. 2000). These studies demonstrate that oxidative damage to individual histone proteins or histone octamers can result in the transfer of oxidative damage to associated DNA via the formation and subsequent decomposition of protein hydroperoxides to reactive radicals, and provide a novel route for the formation of mutagenic lesions in DNA (Luxford, Morin et al. 1999; Luxford, Dean et al. 2000; Luxford, Dean et al. 2002).

The crosslinking of DNA with proteins affect replication, gene activation and normal function of DNA (Gebicki and Gebicki 1999). Exposure of DNA to several proteins peroxidized by radiation-generated hydroxyl free radicals resulted in formation of crosslinks between the macromolecules, detected by retardation and broadening of DNA bands in agarose gel electrophoresis (Gebicki and Gebicki 1999). The degree of crosslinking depended on the concentration and number of hydroperoxide groups and on the duration of interaction DNA with protein. The gel shift of the crosslinked DNA was reversed by proteinase K digestion, which suggested that the protein hydroperoxides did not induce DNA strand breaks. Therefore, the crosslinking of DNA with proteins mediated by protein hydroperoxides is spontaneous and probably covalent, and it may be assisted by transition metals (Drygin 1998; Gebicki and Gebicki 1999). However, the covalent attachment of proteins or peptides on DNA would affect DNA replication and gene transcription (Sastry and Ross 1998).

1.8 Amino acid, peptide and protein radicals

Formation and reactions of amino acid, peptide and protein radicals

The formation and fate of protein radicals has been studied for the last 100 years. Protein radicals formed directly from ROS-related reactions may have potential to propagate radical reactions to other molecules. Table 1.6 lists some of the reactions of radicals formed in amino acids, peptides and proteins. The symbols LZ and Mb refer to lysozyme and myoglobin, and LZTrp and LZTyr to the corresponding lysozyme amino acids (Gebicki, unpublished work).

Table 1.6 Selected reactions of amino acid, peptide and protein radicals

Radical from	Reaction with	Ref
Trp, Tyr, Met, His,LZTrp, LZTyr	Trolox C	(Bisby, Ahmed et al. 1984)
Trp	ascorbate, TMPD, phenols, Trolox C, thiols	(Jovanovic and Simic 1985)
Trp, LZTrp, LZTyr	phenols, α-tocopherol, sesamol, ascorbate, uric acid	(Hoey and Butler 1984)
LZTrp	$O_2^{\bullet-}$, ascorbate	(Santus, Patterson et al. 2000)
Tyr	$O_2^{\bullet-}/HO_2^\bullet$, O_2	(Cudina and Josimovic 1987)
Trp	$O_2^{\bullet-}$	(Josimovic, Jankovic et al. 1993)
Cys	O_2, Tyr	(Al-Thannon, Barton et al. 1974; Prutz, Butler et al. 1986)
GSH	O_2, ascorbate	(Tamba, Simone et al. 1986)
Tyr	Tyr, Cys, O_2, GSH, ascorbate, $O_2^{\bullet-}$	(Boguta and Dancewicz 1983; Prutz, Butler et al. 1983; Jin, Leitich et al. 1993; Sturgeon, Sipe et al. 1998)

Trp	O_2	(Candeias, Wardman et al. 1997)
Trp, Tyr	flavonoids	(Filipe, Morliere et al. 2002)
LZTrp, LZTyr, N-Ac-Trp-OMe N-Ac-Tyr-OMe	GSH	(Nauser, Koppenol et al. 2005)
Egg albumin	ascorbate	(Miyazaki, Yoshimura et al. 1995)
Trp, Tyr in amino acids, peptides, proteins	NO	(Eiserich, Butler et al. 1995)
metmyoglobin	BSA, γ-globulin, β-lactoglobulin	(Ostdal, Skibsted et al. 1997)
BSA	urate, poly(Glu-Ala-Tyr), linoleic acid	(Ostdal, Davies et al. 2002)
Histone-OOH + Cu^{2+}	purine and pyrimidine nucleobases, DNA	(Luxford, Dean et al. 2000)
MbTrpOO$^\bullet$ MbTyrO$^\bullet$	amino acids, peptides, proteins, GSH, ascorbate, Trolox C, vitamin E, urate	(Irwin, Ostdal et al. 1999)
Hydroperoxides from histone, melittin, lysozyme, lysine + Cu^{2+}	DNA, 2'deoxyguanosine	(Luxford, Morin et al. 1999)
BSA hydroperoxide Fe^{2+}-EDTA	Trolox C, ascorbate and derivatives	(Morgan, Dean et al. 2004)
Hydroperoxides in cell proteins and $(Leu)_3$	cell constituents	(Headlam and Davies 2003)

(From Gebicki, unpublished work)

The predominant mechanism of formation of protein radicals *in vivo* accepted at present is by reaction similar to reaction 1.27, where the biological oxidant is $HO^\bullet$ formed in a site-specific Fenton reaction, or the alkoxyl, peroxyl, thiyl or carbon-centred radicals (Davies and Dean 1997; Davies 2005). Reaction of most radicals with large peptides and proteins results in the initial formation of carbon-centred radicals at either side chains or α-carbon sites (Hawkins and Davies 2001). The identity of the protein radical and the course of its reactions depend on factors such as the nature of the oxidative stress, the point of attack by the initiating radical, any translocations to new sites before reaction with solutes, lifetime of the protein radical, the size and shape of the protein involved and the presence of modifying species such as dioxygen, radical-scavenging solutes, or transition metals (Gebicki, unpublished work).

The major destiny of carbon-centred radicals formed on proteins is dimerisation in the absence of O_2, and reaction to form peroxyl radicals with O_2 (Neta, Huie et al. 1990). Peroxyl radicals can also be produced in the absence of O_2, from metal ion-catalysed decomposition of hydroperoxides (reaction 1.32 and 1.33). Peroxyl radicals undertake a number of reactions that result in the formation of carbonyl group, alcohols and hydroperoxides (Davies, Fu et al. 1995; Davies 1996). Alkoxyl radicals can be produced from peroxyl radicals via one-electron reduction of alkyl hydroperoxides or dialkylperoxides. Thiyl radicals are readily generated by either hydrogen abstraction from a free thiol group or by cleavage of disulphide linkages. Thiyl radicals react rapidly with O_2 to form peroxyl radicals, $RSOO^\bullet$, which can give rise to oxylacids and sulphinyl ($RSO^\bullet$) radicals (Davies, Forni et al. 1987).

Of particular interest for studies of reactions of protein radicals is the location of the unpaired electron at the moment of reaction with a biomolecule, because the identity, reactivity and accessibility of the protein radical to solutes ultimately determine its potential to propagate further reactions. There are significant difficulties in studies of the quantitative aspects of the formation and reactions of protein radicals. In the case of highly reactive ROS such as the $HO^\bullet$, the nature and location of the protein radical involved is largely unknown when it is generated randomly, as in exposure to ionizing radiations (Gebicki, unpublished work). Estimation of the possibility of a protein radical that will react with a particular cell component can be achieved at different levels of confidence.

1.9 Aims of this study

I. *In vitro* studies

The formation and reactions of protein hydroperoxides in complex biological systems subjected to ROS have been widely investigated; on the other hand, antioxidants which could protect against protein hydroperoxides in cells were rarely studied. The antioxidants, which can scavenge protein radicals in earlier stages of ROS-induced damage, should be investigated.

1. Testing the effect of a range of radical species on protein/amino acid hydroperoxide formation;

2. Evaluating the protein hydroperoxide- induced ascorbic acid oxidation;

3. Screening a range of antioxidants to prevent or reduce protein hydroperoxide-induced ascorbic acid oxidation.

II. In cell culture studies

The aim of this project is to investigate the role of ascorbate and related compounds in protecting cells from oxidative stress induced by protein oxidation. Oxidized proteins pose a significant hazard to cells, so that proof of protection by the vitamin would provide a mechanism for its antioxidant action in living organisms, which is still unclear. In addition, compounds sparing ascorbate in cells will act as additional line of antioxidant defense. Identification of such compounds will lead to development of new therapies for humans suffering from the wide range of diseases known to be caused by oxidative stress.

1. Testing if intracellular ascorbate can inhibit formation of protein peroxides in cells exposed to ROS;

2. Testing if intracellular glutathione can effect formation of protein peroxides in cells exposed to ROS;

3. Testing and evaluation of selected protein antioxidants able to inhibit damage to cells.

CHAPTER 2

MATERIALS & STANDARD METHODS

2.1 Materials

2.1.1 Reagents

Reagents	Source
2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS)	Sigma, St. Louis, MO, USA
Acetic acid, glacial	Merck Darmstadt, Germany
N-acetylcysteine (NAC)	Sigma, St. Louis, MO, USA
Ascorbic acid	Sigma, St. Louis, MO, USA
Ascorbate oxidase	Sigma, St. Louis, MO, USA
Alanine (Ala)	Sigma, St. Louis, MO, USA
Ammonium ferrous sulphate ($NH_4FeSO_4 \cdot 6H_2O$)	Sigma, St. Louis, MO, USA
Apigenin (4',5,7-Trihydroxyflavone)	Sigma, St. Louis, MO, USA
Arginine (Arg)	Sigma, St. Louis, MO, USA
Asparagine (Asn)	Sigma, St. Louis, MO, USA
Aspartic acid (Asp)	Sigma, St. Louis, MO, USA
Bilirubin	Sigma, St. Louis, MO, USA
Bio-Rad Protein reagent	Bio-Rad Lab., Richmond, CA, USA
Bovine serum albumin (BSA) (Fraction V)	Sigma, St. Louis, MO, USA

L-buthionine sulfoximine (BSO)	Sigma, St. Louis, MO, USA
Calcium dichloride	BDH Chemicals, Sydney, Australia
α-Casein	Sigma, St. Louis, MO, USA
Catalase (18,230 unit/ml)	Roche Diagnostics, Sydney, Australia
Chelex 100 (50-100 mesh sodium form)	Bio-Rad Lab., Richmond, CA, USA
Chymotrypsin	Sigma, St. Louis, MO, USA
Cysteine (Cys)	Sigma, St. Louis, MO, USA
Dimethylsulfoxide (DMSO)	Sigma, St. Louis, MO, USA
3-(4,5)-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)	Sigma, St. Louis, MO, USA
5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Ellman's reagent)	Sigma, St. Louis, MO, USA
Di-sodium hydrogen orthophosphate	BDH Chemicals, Sydney, Australia
Dithiothreitol (DTT)	Sigma, St. Louis, MO, USA
Dodecyltrimethyl ammonium chloride	Sigma, St. Louis, MO, USA
Epigallocatechin gallate	Aldrich, Milwaukee, MI, USA
Ethanol	BDH Chemicals, Sydney, Australia
Ethylenediaminetetracetic acid (EDTA)	Sigma, St. Louis, MO, USA
Ferric ammonium sulfate	BDH Chemicals, Sydney, Australia
Ferrous ammonium sulfate	BDH Chemicals, Sydney, Australia
Ferrozine	BDH Chemicals, Sydney, Australia
Gallic acid	Sigma, St. Louis, MO, USA
Glycine (Gly)	BDH Chemicals, Sydney, Australia
Glutamic acid (Glu)	Sigma, St. Louis, MO, USA

Glutamine (Gln)	Sigma, St. Louis, MO, USA
L-Glutamine (200 mM)	Trace Scientific, VIC, Australia
Glutathione (GSH)	Sigma, St. Louis, MO, USA
Guanidine hydrochloride	Sigma, St. Louis, MO, USA
HEPES	AnalaR, BDH Chemicals, Australia
Histidine (His)	Sigma, St. Louis, MO, USA
Human serum albumin (HSA)	Sigma, St. Louis, MO, USA
Hydrochloric acid, conc.	Sigma, St. Louis, MO, USA
4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy (TEMPOL)	Aldrich, Milwaukee, MI, USA
6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)	Aldrich, Milwaukee, MI, USA
Isoleucine (Ile)	Sigma, St. Louis, MO, USA
Leucine (Leu)	Sigma, St. Louis, MO, USA
Lysine (Lys)	Sigma, St. Louis, MO, USA
Lysozyme	Boehringer Mannheim, Germany
Magnesium dichloride	BDH Chemicals, Sydney, Australia
Methionine (Met)	Sigma, St. Louis, MO, USA
Methanol	Merck Darmstadt, Germany
N-*tert*-butyl-α-phenylnitrone (PBN)	Aldrich, Milwaukee, MI, USA
Nitric acid, conc.	Merck Darmstadt, Germany
Orthophosphoric acid	AJAX Chemicals, Sydney, Australia
Penicillin/Streptomycin (5000IU/ml penicillin G, 5000 μg/ml streptomycin	Trace Scientific, Australia

Perchloric acid (PCA) (70-72%)	Merck Darmstadt, Germany
Potassium chloride	BDH Chemicals, Sydney, Australia
Potassium dihydrogen orthophosphate	BDH Chemicals, Sydney, Australia
Proline (Pro)	Sigma, St. Louis, MO, USA
Serine (Ser)	Sigma, St. Louis, MO, USA
Silibinin	Aldrich, Milwaukee, MI, USA
Sodium acetate	BDH Chemicals, Sydney, Australia
Sodium azide	Sigma, St. Louis, MO, USA
Sodium chloride	BDH Chemicals, Sydney, Australia
Sodium dihydrogen orthophosphate	BDH Chemicals, Sydney, Australia
Sodium formate	BDH Chemicals, Sydney, Australia
Sodium hydroxide	BDH Chemicals, Sydney, Australia
di-sodium orthophosphate	BDH Chemicals, Sydney, Australia
Tri-sodium orthophosphate	Ajax Chemicals, Sydney, Australia
Sodium phosphate	Sigma, St. Louis, MO, USA
Sulphuric acid, conc.	BDH Chemicals, Sydney, Australia
Superoxide dismutase (SOD) (2,600 unit/mg)	Roche Diagnostics, Sydney, Australia
2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO)	Sigma, St. Louis, MO, USA
Threonine (Thr)	Sigma, St. Louis, MO, USA
Tetraocylammonium bromide	Sigma, St. Louis, MO, USA
Trichloroacetic acid (TCA)	Aldrich, Milwaukee, MI, USA
Trypan blue	Sigma, St. Louis, MO, USA
Xylenol orange, sodium salt	Sigma, St. Louis, MO, USA

2.1.2 Media

RPMI-1640	Trace Scientific, Australia
Fetal calf serum (FCS)	Trace Scientific, Australia

2.1.3 Culture vessels, serological pipettes and centrifuge tubes

96-well microtitration plates	Greiner, Frickenausen, Germany
60-mm^2 diameter petri dishes	Falcon, NJ, USA
25-cm^2 flask with vented cap	Iwaki, Chiba, Japan
75-cm^2 flask with vented cap	Iwaki, Chiba, Japan
2 ml sterile plastic pipettes	Sterilin, Staffordshire, UK
10 ml sterile plastic pipettes	Sterilin, Staffordshire, UK
50 ml sterile plastic pipettes	Sterilin, Staffordshire, UK
10 ml sterile centrifuge tubes	Iwaki, Chiba, Japan
50 ml sterile centrifuge tubes	Iwaki, Chiba, Japan

2.1.4 Preparation of glassware and plasticware

All glassware used in handling and storage of solutions was soaked in detergent overnight before rinsing with water, and then cleared by heating for several hours in conc. nitric acid. After cooling, the glassware was washed with distilled water purified in a four-stage Milli Q system (Sydney, Australia) equipped with a 0.2 μm-pore-size final filter, and dried at 180 ^{o}C over overnight. For cell culture experiments, the glassware was soaked in detergent,

and then rinsed in RO water and Milli-Q water before drying. The glassware, Eppendorf tubes and pipette tips were sterilized by autoclaving at 121 $^{\circ}$C, 100 kPa for 20 minutes.

2.1.5 Solutions and Buffers and Media preparation

(I) *In vitro* studies

(A) Fricke dosimeter stock solution

Ammonium ferrous amminosulfate (0.2 g, final concentration: 2 mM), 0.03 g NaCl (1 mM), and 11 ml conc. H_2SO_4 (0.4 M), made up to 0.5 L Milli-Q water (notice: ferrous added after the acid). Stored in a light-protected bottle at 4 $^{\circ}$C for weeks.

(B) Ascorbic acid stock solution

Ascorbic acid stock solution (0.5 mM): fresh made every day. 0.0176 mg ascorbic acid was dissolved in 10 ml Milli-Q water. Diluted 10 in Milli-Q water. Stored cold until use.

(C) Catalase solution

Stock solution: 50 μl of catalase suspension (18,230 units/ml) added to 1ml Milli-Q water. Diluted stock: catalase concentration: 868 units/ml. Hydrogen peroxide decomposed in the solutions containing 26 units of catalase per ml of solution.

(D) 0.2 M pH 7.0 Sodium phosphate buffer

NaH_2PO_4 (0.2 M) under pH meter titrated with 0.2 M Na_2HPO_4 to pH 7.0, and then treated with Chelex 100 (50-100 mesh sodium form) overnight for the removal of any contaminating transition metals from solution.

(E) Sodium formate stock solution

Stock 2 M sodium formate solution was buffered with 0.02 M Sodium phosphate to pH 7.0.

(F) Sodium azide stock solution

Sodium azide (1 M) solution was made in Milli-Q water.

(G) FOX assay solutions

5mM XO: xylenol orange (XO) was made up in 110 mM $HClO_4$ and stored at room temperature.

5mM Ferrous solution: Ammonium ferrous sulphate was made up in 110 mM $HClO_4$ and stored at 4^oC.

6M guanidine HCl: made up in Milli-Q water.

(H) Ferrozine assay solutions

Sodium acetate-acetic acid buffer pH 4.3:

Sodium acetate (0.1 M): 0.82 g anhydrous sodium acetate powder dissolved into 100 ml Milli-Q water. To make the buffer 16.6ml 0.1M sodium acetate was mixed with 83.4 ml 0.1 M acetic acid to pH 4.3.

10mM ferrozine: Solution contained take 0.049 g ferrozine in 10 ml Milli-Q water.

5mM Fe (III): Solution contained 0.024g Fe (III) in 10 ml 110 mM $HClO_4$ solution.

(II) Cell culture studies

(A) HL-60 cell experimental solutions

Cell culture media

Serum-containing media

RPMI-1640 was supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and 100 units/ml penicillin and 100 μg/ml streptomycin. The pH of the medium, indicated by phenol red, was adjusted to 7.4 with 7.5 % $NaHCO_3$.

Serum-free media: RPMI-1640 (without L-glutamine) only.

All solution were stored at 4 ^{o}C and handled under sterile conditions in a sterile laminar flow hood.

Phosphate-buffered saline (PBS)

137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 10mM Na_2HPO_4 were dissolved in Milli-Q water. Afterwards, the pH was adjusted to 7.4 and the buffer solution was sterilized by autoclaving and stored at 4 ^{o}C.

*Cell lysis solution***:** made up 60 % methanol and 1 mM EDTA (pH 8.0), and stored at 4°C.

HEPES buffer

Buffer contained 15 mM HEPES pH 7.6, 135 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 0.1 mM DTT in Milli-Q water.

(B) Ascorbic acid uptake solutions

Phosphate buffer for ascorbic acid

Dissolved 0.1 M KH_2PO_4, 4 mM Na_2HPO_4, 0.5 mM EDTA in distilled water and adjusted to pH 5.6 by HCl , then sterilized by autoclaving and stored at room temperature. Solutions of ascorbic acid were prepared daily.

Sodium phosphate buffer for ascorbate oxidase

NaH_2PO_4 (4mM) was titrated under pH meter with 4 mM Na_2HPO_4 to pH 5.6, 0.01 % BSA added and solution stored at 4 ^{o}C.

Ascorbate oxidase solution

A stock solution of ascorbate oxidase was made by diluting 382.5 units of ascorbate oxidase in 2.5 ml of sodium phosphate buffer. This stock solution was divided into aliquots of 250 μl in Eppendorf vials and stored at -20 ºC in the dark.

(C) MTT assay solutions

MTT stock solutions: MTT was dissolved in 1 × PBS at 5 mg/ml, 0.22 μm-filter sterilized and stored in the dark at 4°C. MTT stock solutions were discarded after one month.

(D) HPLC assay solutions

Mobile phase buffer: The 25/75 methanol/water (v/v) mobile phase was made up of 0.05 M sodium phosphate, 0.05 M sodium acetate and 189 μM dodecyltrimethylammonium chloride in 400-500 ml water and 3.66 μM tetraoctylammonium bromide in 250 ml

methanol. The two solutions were mixed and diluted to a final volume of 1 litre with water. The tetraoctylammonium bromide was dissolved in the methanol first, because of its insolubility in water. The pH of the final solution was adjusted to 4.8 with phosphoric acid. The mobile phase was filtered through 0.22 μm Durapore hydrophilic filters (Millipore Corp) prior to use.

Methanol solution: methanol was filtered through 0.22 μm Durapore hydrophilic filters (Millipore, Corp) prior to use.

(E) Bradford assay solutions

Bradford reagent: 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol in 100 ml 85 % (w/v) phosphoric acid. Diluted to 1 liter when the dye has completely dissolved, and the solution filtered just before use.

(F) Ellman's assay solution

Phosphate–EDTA buffer: 100 mM sodium phosphate, 5mM EDTA, pH 7.5 stored at room temperature.

10 mM DTNB stock solution: 0.0396 g DTNB in 10 ml phosphate-EDTA buffer, stored at 4 ºC and discarded after two weeks

3 % PCA: 87.5 ml of 70 % PCA stock solution to make final volume of 2 liters.

10 mM GSH solution: prepared immediately before use in cold 3 % PCA.

0.5 M tri-sodium phosphate: 2.63 g $Na_3PO_4 \cdot 12H_2O$ in 100 ml Milli-Q water.

2.2 Methods

2.2.1 Determination of dose rate of the cobalt-60 gamma radiation source

Thee energy dose rate of the ^{60}Co source was measured by Fricke dosimetry (Fricke and Morse 1927). This involves the spectrophotometric measurement at 304 nm of samples of ferrous sulphate solution irradiated with a range of gamma doses.

Aliquots of 1.5ml stock Fricke Dosimetry solution in eppendoff tubes were irradiated for 0, 1, 2, 3, and 4 minutes and three tubes removed at a time. Absorbance at 304nm was measured against non-irradiated samples and the standard curve was generated used for dose rate calculation. The dose rate was calculated according to the formula:

$$\text{Dose rate } \textbf{Error! Bookmark not defined.} = 286 \times (dA/t)$$

unit: Gy /min

Where dA = absorbance of irradiated sample, t is in minutes, and 1Gy (gray) = 1 J/kg.

(I) *In vitro* studies

2.2.2 Ascorbic acid measurement

Pure ascorbic acid is a white crystalline solid, and very soluble in water. Since ascorbic acid has pK_{a1} in 4.25 and pK_{a2} in 11.8, a mono-anion form is the favoured form at physiological pH. Therefore, we used the name ascorbate in pH 7.0 solution, and ascorbic acid in acid solution (Halliwell and Gutteridge 1999). The real form in the test-tube or cell culture studies is ascorbate, not ascorbic acid. However, for the following experiments, the

level of ascorbic acid was mostly measured in the acid solution. Hence, I used the name of ascorbic acid from now on.

2.2.2.1 Ultraviolet spectrophotometry

Ascorbic acid absorbance in phosphate buffer, pH 7.0, was measured at 265 nm, corresponding to the typical absorption peak of L-ascorbic acid in neutral solution. Ascorbic acid absorbance in PCA was measured at 244.5nm, corresponding to the typical absorption peak of L-ascorbic acid in acidic solution on a Shimadzu UV-1201 spectrophotometer (Kyoto, Japan)(Yang, Lee et al. 2003; Zeng, Martinuzzi et al. 2005).

2.2.7.2 Ferrozine assay

Samples of 200 µl of test solutions and 225 µl water were mixed with 500 µl 0.1 M sodium acetate-acetic acid buffer pH 4.0, 50 µl 5 mM Fe (III)in the same buffer and 25 µl 10 mM ferrozine; after standing for 10 minutes in room temperature purple color was fully developed. The absorbance at 562 nm was read in a Shimadzu UV mini 1240 spectrophotometer. Molar absorption coefficient of the Fe^{2+}- ferrozine complex was reported to be $2.79{\times}10^4$ l mol^{-1} cm^{-1} (Stookey 1970).

2.2.3 Irradiation of ascorbic acid, protein and amino acid solutions

Samples were placed in 1.5 ml eppendorf tubes or Oxford pipettor in the centre sample holder of the ^{60}Co gamma radiation source at Macquarie University and irradiated at room temperature for variable lengths of time. At times, irradiation was carried out under nitrous oxide or argon to allow hydroxyl radicals formation but prevent the generation of superoxide radicals and hydroperoxide groups. Manipulation of the irradiation systems in

air-saturated and anoxic systems yields specific radical formation (Simpson, Narita et al. 1992). The anoxic conditions were performed by pre-gassing of samples with argon and nitrous oxide for 2 hours in an oxford (Monoject Scientific, St. Louis, MO, U.S.A.) volume pipettor and sealing before irradiation. At the end of the radiation time, the sample was incubated for 10 minutes at room temperature with 30 μl dilute catalase (26 units) per ml solution to remove radiation generated hydrogen peroxide, before further experimental uses (Davies 2000). The solution of ascorbic acid with proteins were then acidified to final concentration of 2 M perchloric acid (PCA), kept on ice for 20 min and then centrifuged at 16,500 rpm (11,500g, Zentrifugen Hettich Universal 10R, Hettich, Tuttlingen, Germany) for 20 min at 4 °C. The ascorbic acid concentration of the supernatant of each sample was analyzed using an UV spectrophotometer.

2.2.4 Superoxide dismutase treatment

Under air-saturated condition, superoxide anion ($O_2^{\bullet-}$) was formed in irradiated solutions. Before irradiation, 50 unit/ml SOD was added into sample solutions to dismutate the $O_2^{\bullet-}$ generated (Nappi 1997).

2.2.5 Sodium formate treatment

0.2 M sodium formate was added to ascorbic acid or protein/ascorbic acid solution before irradiation to give superoxide anion radical formation.

2.2.6 Azide treatment

Different concentration of ascorbic acid solutions were irradiated by gamma irradiation with different doses (39.7 Gy/min) in the presence or absence of 0.1 M azide. The different proteins, lysozyme and chymotrypsin, were also added in some samples to ascorbic acid solution before the irradiation.

2.2.7 FOX assay of protein hydroperoxides

To each sample solution, 55 µl of 0.5 M PCA, 25 µl of 5 mM XO and Fe^{2+} were added, and then the mixture incubated in the dark for 40 to 60 minutes. Each sample was analyzed using an absorbance maximum of λ_{max} at 560 nm by UV spectrophotometer (Gay, Collins et al. 1999).

2.2.8 Antioxidants

A range of antioxidants: TEMPO, TEMPOL, PBN, Rutin, Silibinin, Trolox, ABTS, gallic acid, and epigallocatechin gallate are used against protein radical-induced ascorbic acid oxidation and the formation of protein hydroperoxides.

(II) Cell culture studies

2.2.9 HL-60 cell culture

The human myeloid leukemia cells line HL-60 can transport the oxidized form of ascorbic acid, dehydroascorbic acid (DHA), and accumulate reduced ascorbic acid via glutathione – independent pathway (Guaiquil, Farber et al. 1997). The HL-60 cells were grown as suspension in RPMI-1640 containing 10 % (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were passaged at 1 $\times10^5$ viable cells/ml every 2 to 4 days, and the cultures were maintained in 75-cm^2 tissue flasks at 37 ^{o}C in a humidified atmosphere of 5 % CO_2/95 % air.

2.2.10 Cell number counting

Trypan blue exclusion assay

40 µl trypan blue was added to the 40 µl cell suspensions, and the numbers of unstained (viable) and stained (dead) cells were counted in more than six repeats using Neubauer Improved haemocytometer under a microscope.

2.2.11 Cell viability assay

MTT assay

Sample cells (200 µl; 5 $\times10^5$ cells/well) were added into 96-microwell plates, 0.25 mg (50 µl of a 5 mg/ml solution) of MTT was added to each well, the plates wrapped in aluminum foil and then incubated for 4 hours in a humidified atmosphere at 37 ^{o}C. Plates were centrifuged at 1000 rpm for 5 min at room temperature and the media was carefully

aspirated. 200 µl of DMSO was then added to each well to dissolve the remaining MTT-formazan crystals. The plates were read immediately at 570 nm on a multiwell scanning spectrophotometer (Microplate Manager [R], Bio-Rad Laboratories, Inc.). All experiments were performed more than three times and the mean absorbance values were calculated.

2.2.12 Irradiation of cells

HL-60 cells in the HEPES buffer or serum-free RPMI-1640 were irradiated in 60-mm diameter petri dishes (the depth of the medium kept within the range 2-5 mm) at room temperature, using a ^{60}Co source with an average dose rate of 39.7 Gy/min. Exact dose rates were measured by a Fricke dosimeter and recalculated when necessary from standard decay tables. Use of pertri dishes increased the surface area of the medium that helps oxygen diffuse to the cells, which can increase the yields of peroxides.

2.2.13 Hydroperoxide assays

G-PCA-FOX assay

Cells (2.5×10^6 cells/ml) were harvested in glass test tubes by centrifugation at 4,500 rpm for 5 min at 4 $^\circ$C, the supernatants were discarded and washed with PBS once. Then 745 µl of 6 M Gu-HCl was added to each tube, the cell pellet was dissolved by vortexing until the solution was clear, and 40 µl of 0.5 M PCA was added. After vortex mixing, 25 µl of 5 mM XO and Fe^{2+} were added to the mixture, followed by 15 µl H_2O, making the total volume to 850 µl. Each tube was covered by parafilm and incubated in dark for 40 to 60 minutes. The absorbance at 560 nm was measured after the removal of insoluble cell debris by

centrifugation. The concentrations of protein hydroperoxides were calculated with the molar absorption coefficient of 36,000 $M^{-1}cm^{-1}$(Gay and Gebicki 2003).

2.2.14 Measurement of intracellular ascorbic acid

2.2.14.1 Sample preparation

After incubation or irradiation, HL-60 cells (5×10^6 cells) were washed by centrifugation with ice-cold phosphate-buffered saline, pH 7.4. Cells pellets were extracted using 0.5 ml of ice-cold 60 % methanol and 1 mM EDTA (pH 8.0). After centrifugation at 16,500 rpm at 4 ^{o}C for 20 minutes, supernatants were stored at -80 ^{o}C until analysis.

2.2.14.2 High performance liquid chromatography (HPLC)

Apparatus

Quantitative HPLC was performed on a reverse phase high pressure liquid chromatograph (Shimadzu HPLC Class 10 Aseries) with a degasser (Model DGU-3A), pump (Model LC-10Ai), injector (Model SIL-10Ai), a multiple wavelength programmable UV/VIS detector (SPD-10A), a guard column (Shimadzu TM, ODS 2cm, Shim-pack) and RP C_{-18} column (250 mm × 4.6 mm i.d., particle size 5 μ) was used. The HPLC system was equipped with the software "Class LC-10AT series version 10" (Shimadzu).

HPLC conditions

The composition of the mobile phase was adapted from Washko (Washko, Hartzell et al. 1989). The mobile phase consisted of 0.05 M sodium phosphate, 0.05 M sodium acetate, 189 μM dodecyltrimethylammonium chloride, and 3.66 μM tetraocylammonium bromide

in 25/75 methanol/ water (v/v), pH 4.8. Column equilibration took approximately 24 hours at the flow rate of 1.0 ml/min, which yields a column back pressure of 180-200 kg/cm^2, and this flow rate was used for all subsequent analyses. The system was cleaned weekly with methanol at a flow rate of 1.0 ml/min for 8-12 hours and then re-equilibrated in the mobile phase. The running time was set at 10 minutes and the column temperature was maintained at 25 ^{o}C. The volume of injection loop was 100 μ. Prior to injection of the sample, the column was equilibrated for at least 30 minutes with the mobile phase flowing through the systems. The eluents were monitored at 212 and 265 nm, and the data were acquired, stored and analyzed with the software Class LC-10AT series version (Shimadzu). Flow rate in all experiments was set at 1.0 ml/min. The ascorbic acid standards were prepared in 60 % methanol/water (v/v) and 1 mM EDTA. To ensure reproducibility at each assay, standards were run before and after every set of measurements.

2.2.15 Protein estimate in HL-60 cells

Bradford methods (Freshney 2000)

The different cell number of HL-60 cells was harvested by centrifugation and washed by 1 × PBS twice. The cell pellets were treated with 100 μl 0.3 N NaOH. 20 μl of each sample solution was added to 1 ml BioRad Dye Bradford reagent. After 5 minutes incubation, A_{595} was measured in a spectrophotometer. The protein content was estimated by comparison of the results with a standard curve generated with know amounts of BSA (Bradford 1976; Stoscheck 1990).

2.2.16 Measurement of intracellular glutathione

Ellman's assay (Ellman 1959; Moss and Swarup 1988)

Cells were harvested by centrifugation and washed by $1 \times$ PBS twice. The cell pellets were treated with 100 µl 3 % PCA, allowed to stand on ice for 10 minutes and then centrifuged for 16,500 rpm for 10 minutes. The pellet was discarded and 100 µl of supernatants were mixed with 50 µl 0.5 M Na_3PO_4, 750 µl 0.1 M NaH_2PO_4/5 mM EDTA pH 7.5 and 100 µl 10 mM DTNB. Each sample solution was incubated for 5 minutes at room temperature. The absorbance at 412 nm was then measured.

GSH standard curve

The GSH solution was prepared immediately before use in cold 3 % PCA, and the GSH concentration adjusted in the range of 1-10 µM. Standard curve was prepared by plotting Δ_{412}/min against concentration of GSH.

Although the Ellman's assay is a measuremet on cells measures the sums total of celluar thiols, GSH and protein thiols, the minor contribution of the protein groups was eliminated before GSH assay by the addition of PCA which removes the celluar proteins (thiol proteins). Therefore, the modified Ellman's assay as used here is a measure of intracellular GSH.

2.2.17 Statistical analysis

Results were presented as mean $\pm$ SD of triplicate samples, and were analyzed using the Student's t test. Values of $p<0.05$ were considered statistically significant. Data were analyzed by using pair-t test followed by Student test.

CHAPTER 3

RESULTS

3.1 Radiation dose and radical yield determination

3.1.1 Gamma irradiation dose rate measurement

The energy dose rates of gamma irradiation were measured by chemical dosimetry. The Fricke dosimetry system (Fricke and Morse 1927) was used to determine the energy dose rate of the source at the positions in which the samples were irradiated (O'Donnell and Sangster 1970). The radiation experiments were performed in two different positions in the source, one in the Eppendorf tubes in a plastic beaker and the other one in an Oxford pipettor. The two positions gave different dose rates in aqueous solutions.

The Fricke's solution consists of ferrous ion, Fe^{2+}, in H_2SO_4 solution, which reacts with the oxidizing radical species produced by the irradiation of water. The chemical equations for Fricke reactions are:

$$H^\bullet + O_2 \rightarrow HO_2{}^\bullet \tag{3.1}$$

$$HO_2{}^\bullet + Fe^{2+} \rightarrow HO_2{}^{\bullet-} + Fe^{3+} \tag{3.2}$$

$$HO_2{}^{\bullet-} + H^+ \rightarrow H_2O_2 \tag{3.3}$$

$$HO^\bullet + Fe^{2+} \rightarrow OH^- + Fe^{3+} \tag{3.4}$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + Fe^{3+} + HO^\bullet \tag{3.5}$$
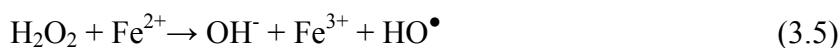
The Fe^{3+} produced was measured at 304 nm by a spectrophotometer (O'Donnell and Sangster 1970).

Fricke Dosimeter solution was irradiated by Co^{60} gamma irradiation for 0 to 4 minutes in different containers. Using the irradiation time for x-axis and the reading of A^{304} for y-axis, standard curves were generated for the two different containers. The dose rate in Gy/min can be calculated by the equation: $286 \times (\Delta A/min)$ where 1 gray (Gy) = 1 J/kg.

(A) Samples in eppendorf tubes in a plastic beaker (Fig. 3.1)

Dose Rate (4min)	$286 \times (0.548)/4= 39.182$ Gy/min
Dose Rate (3min)	$286 \times (0.421)/3= 40.135$ Gy/min
Dose Rate (2min)	$286 \times (0.257)/2= 36.751$ Gy/min
Average	39.7 Gy/min

(B) Samples in oxford pipettor (Fig. 3.2)

Dose Rate (4min)	$286 \times (0.548)/4= 50.765$ Gy/min
Dose Rate (3min)	$286 \times (0.421)/3= 50.972$ Gy/min
Dose Rate (2min)	$286 \times (0.257)/2= 54.531$ Gy/min
Average	52.1 Gy/min
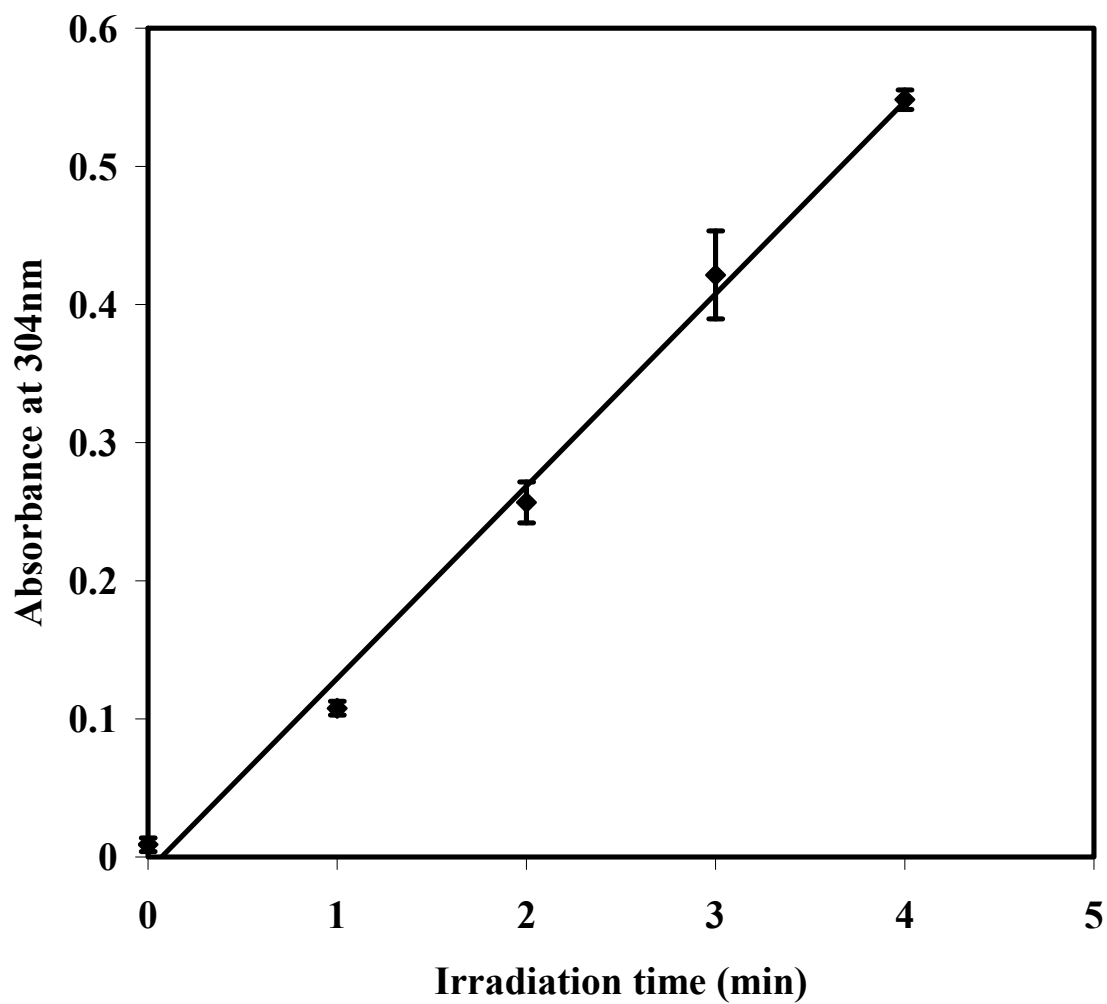
Figure 3.1 Standard curve of radiation dose rate in eppendorf tubes in a plastic beaker by Fricke dosimeter assay

Experimental details are in Section 3.1.1. The coefficient of the curve is y=0.1392x+0.0099, R^2=0.9941. The data points were derived from three experiments, with error bars (N=3, ± SD).
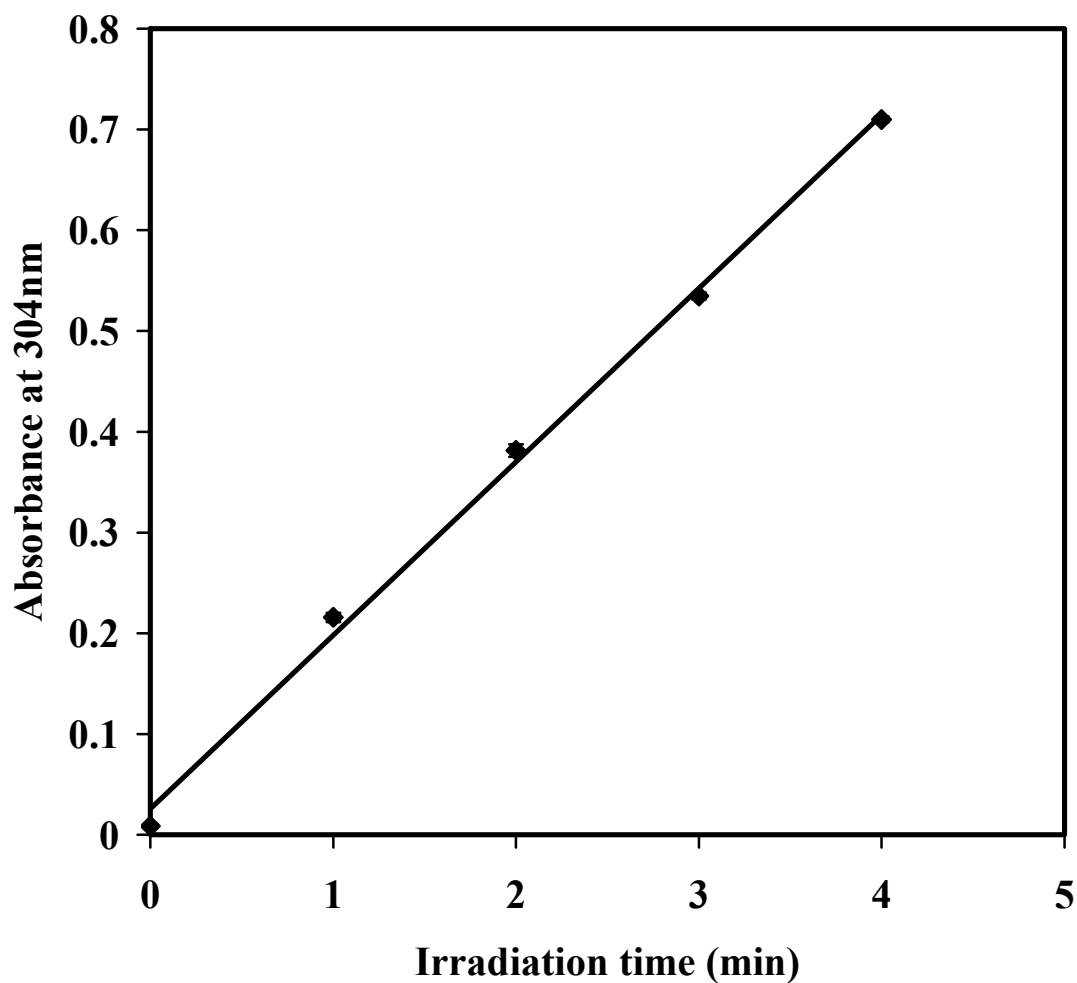
Figure 3.2 Standard curve of radiation dose rate in oxford pipettor by Fricke dosimeter assay

Experimental details are in Section 3.1.1. The coefficient of the curve is y=0.1722x+0.0257, R^2=0.9973. The data points were derived from three experiments, with error bars (N=3, ± SD) which were too small to show.

3.1.2 The amounts of radicals generated by gamma irradiation in different containers

The amount of hydroxyl and superoxide radicals generated by 0 to 120 seconds irradiation was calculated by the equation: $0.1037 \times G \times Dose$ (in Gy). G value for $HO^\bullet$ is 2.7 and for $O_2^{\bullet-}$ is 3.2 (Table 1.3). The results are shown in Figure 3.3 and 3.4 and summarized in Table 3.1.

Table 3.1 The yield of radicals in water after gamma irradiation in different containers

Irradiation time (second)	Radical yield (µM)			
	eppendorf tube		oxford pipettor	
	$HO^\bullet$	$O_2^{\bullet-}$	$HO^\bullet$	$O_2^{\bullet-}$
0	0	0	0	0
30	5.6	6.6	7.3	8.6
60	11.1	13.2	14.6	17.3
90	16.7	19.8	21.9	25.9
120	22.2	26.4	29.2	34.6
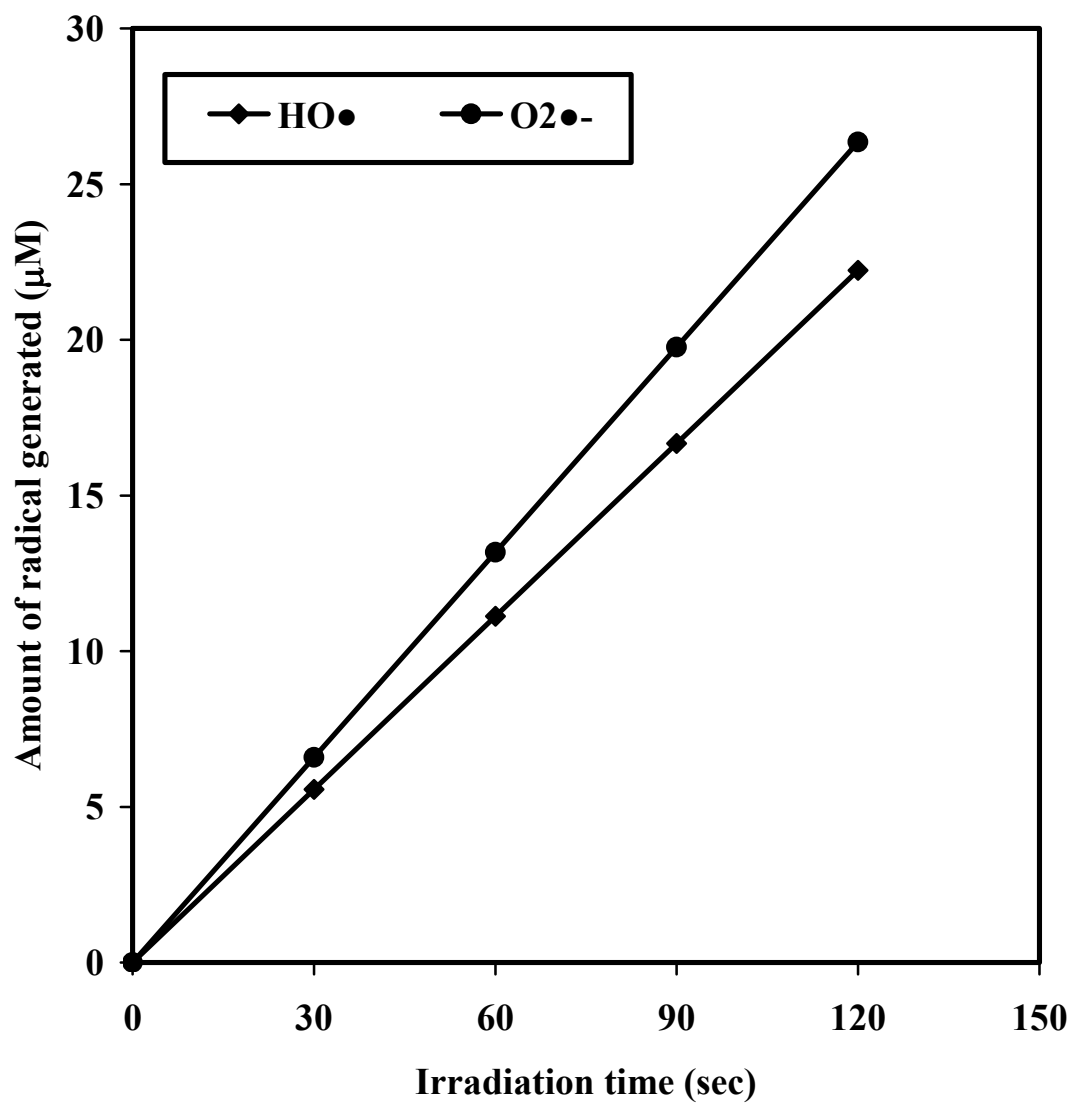
Figure 3.3 The amounts of hydroxyl and superoxide radicals generated by gamma irradiation in eppendorf tubes
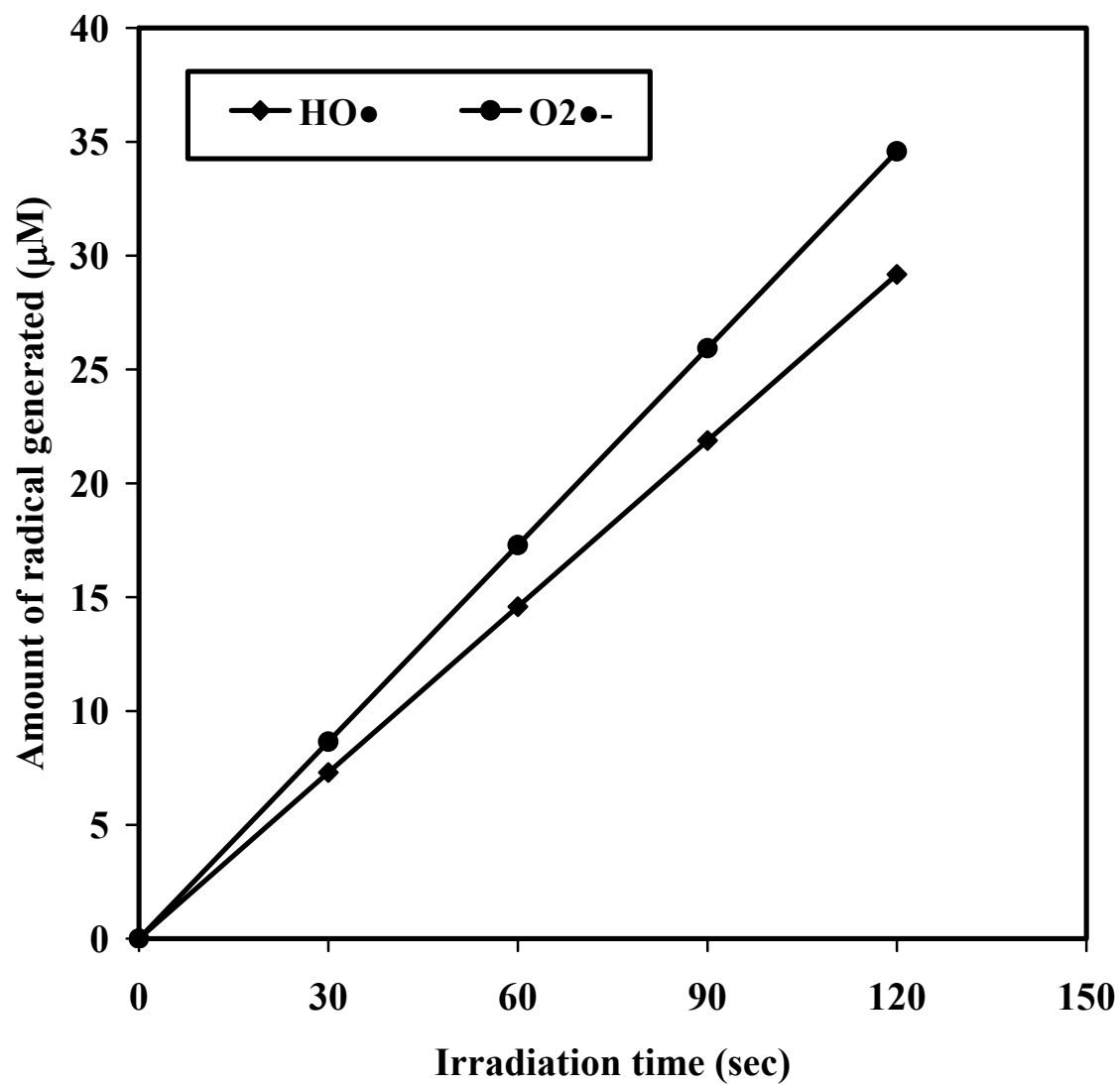
Figure 3.4 The amounts of hydroxyl and superoxide radicals generated by gamma irradiation in the oxford pipettor

SECTION (I) IN VITRO STUDIES

3.2 Effect of γ-irradiation generated free radicals in ascorbic acid oxidation

3.2.1 Ascorbic acid standard curve by ultraviolet spectrophotometer

In neutral solution

Increasing volumes of 0.5 mM ascorbate stock solution were used to make final volume 1 ml in 20 mM phosphate buffer pH 7.0. The final concentration range of ascorbate was 0-50 µM. The standard curve was produced by plotting A_{265}, which is absorbance of λmax of ascorbate in neutral solution, against the concentration of ascorbate (Fig. 3.5). The molar absorption coefficient $\varepsilon = 1.24 \times 10^4$ $M^{-1}cm^{-1}$ was calculated form the graph. In 1990, Buettner reported the molar absorption coefficient of ascorbate in pH 7.4 at 265 nm was 1.45×10^4 $M^{-1}cm^{-1}$ (Buettner 1990).

In acidic solution

Increasing volumes of 0.5 mM ascorbic acid stock solution were used to make final volume 1.23 ml in 20 mM phosphate buffer pH 7.0. The concentration range of ascorbic acid was 0-40 µM. After addition of 270 µl conc. PCA, the final volume was 1.5 ml. The standard curve was produced by plotting $A_{244.5}$, which is absorbance of λmax of ascorbic acid in acid solution, against the concentration of ascorbic acid (Fig. 3.6). The molar absorption coefficient $\varepsilon = 8.23 \times 10^3$ $M^{-1}cm^{-1}$ was calculated from the graph.
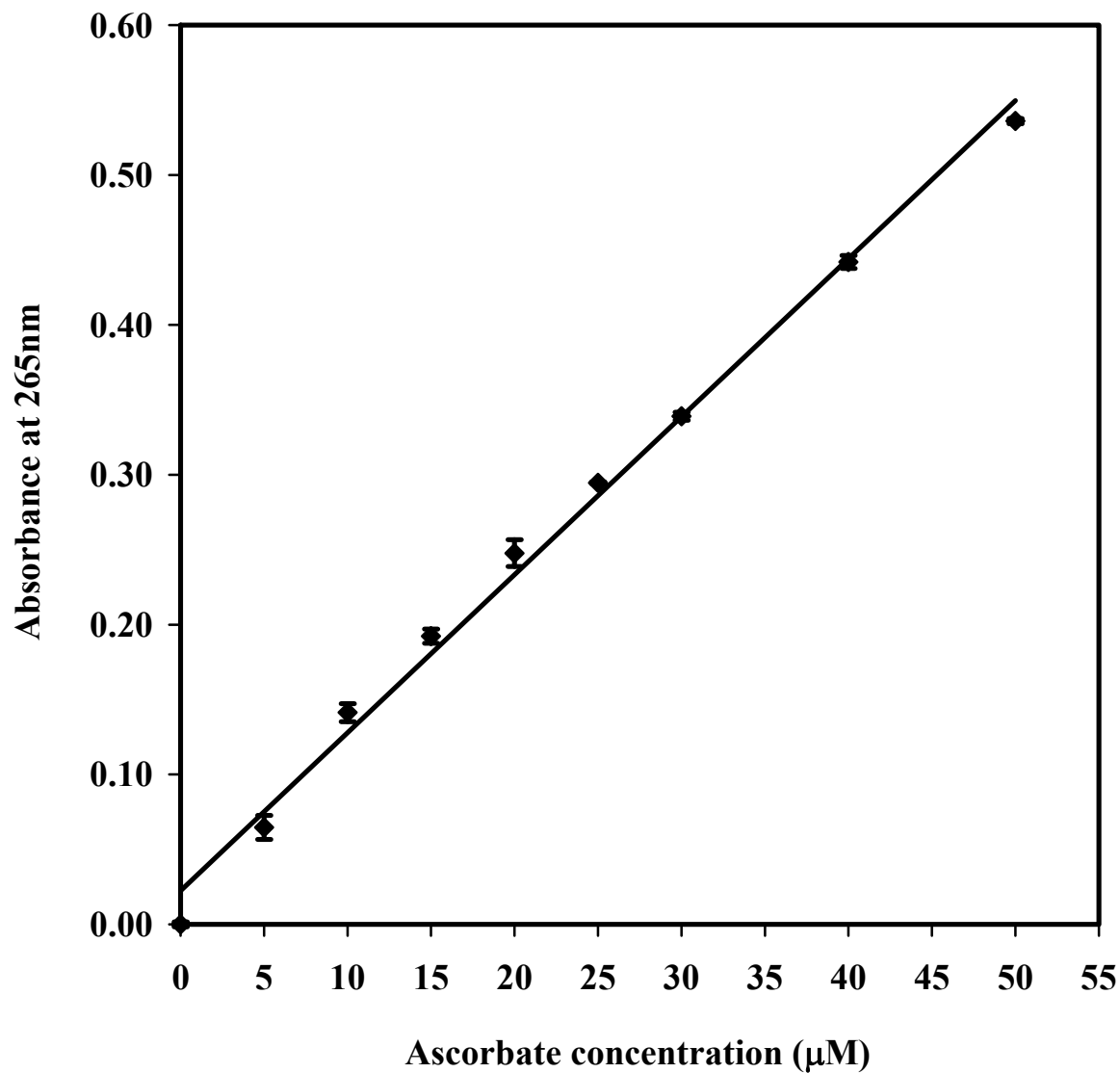
Figure 3.5 Standard curve of ascorbate in phosphate buffer

The standard curve was generated by plotting ΔA_{265} against the concentration of ascorbate. Experimental details are in Section 3.2.1. The molar absorption coefficient was $\varepsilon = 1.24 \times 10^4$ M^{-1} cm^{-1}. The data points were derived from two experiments, with error bars (N=6, $\pm$ SD).
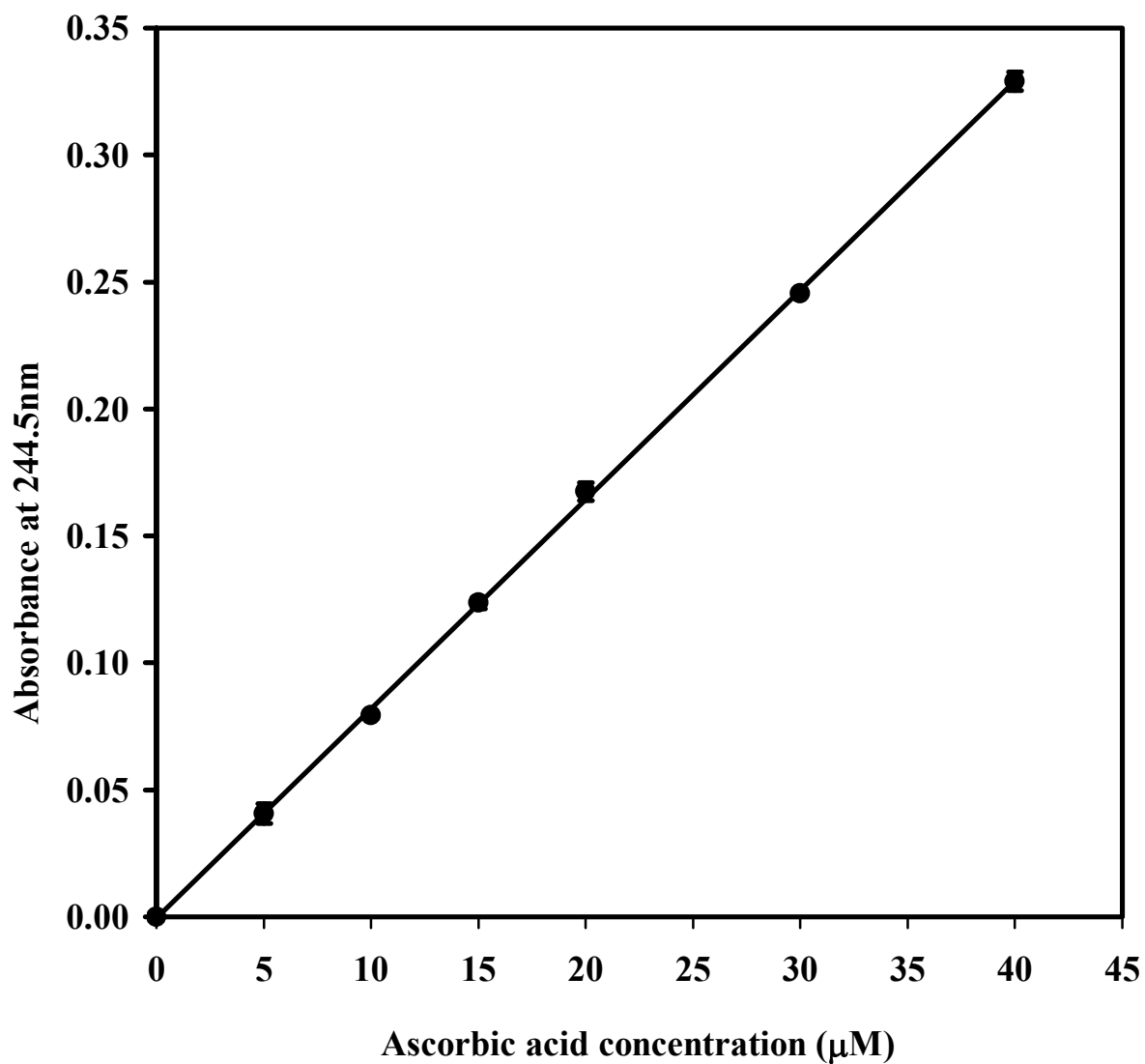
Figure 3.6 Standard curve of ascorbic acid in 2M PCA

The standard curve was generated by plotting $\Delta A_{244.5}$ against the concentration of ascorbic acid. Experimental details are in Section 3.2.1. The molar absorption coefficient was $\varepsilon= 8.23 \times 10^3$ M $^{-1}$ cm^{-1}. The data points were derived from two experiments, with error bars which is too small and can not clear shown in this figure (N=6, $\pm$ SD).

3.2.2 The effect of gamma irradiation on different concentrations of ascorbic acid under air

The 0 to 45 μM ascorbate solutions in 20 mM phosphate buffer pH 7.0 were irradiated by gamma irradiation for 0, 30, 60, 90 and 120 seconds, and then 270 μl conc. PCA was added to final concentration of 2 M PCA in 1.5 ml Eppendorf tubes. The absorbance of ascorbic acid was measured at 244.5 nm. Figure 3.7 was generated by plotting the oxidized ascorbic acid concentration after different irradiation times (calculated by using $\varepsilon = 8.23 \times 10^3$ M^{-1} cm^{-1}) against the initial concentration of ascorbic acid (gamma dose: 0-79.4 Gy).

The result show that the level of ascorbic acid oxidation increased with increasing gamma dose for different ascorbic acid concentration groups (Fig. 3.7). However, the amount of oxidized ascorbic acid reached a maximum which depended on ascorbic acid concentration and radiation dose. After 120 second irradiation, 22.2 μM HO$^\bullet$ and 26.4 μM O$_2^{\bullet-}$ were generated (Table 3.1), resulting in oxidation of all the ascorbate in solution Therefore, the 30 μM ascorbic acid might be enough to scavenge all radicals generated in the irradiation. Since we planned to work below the scavenging concentration, 20 μM ascorbic acid was selected for further experiments.

115
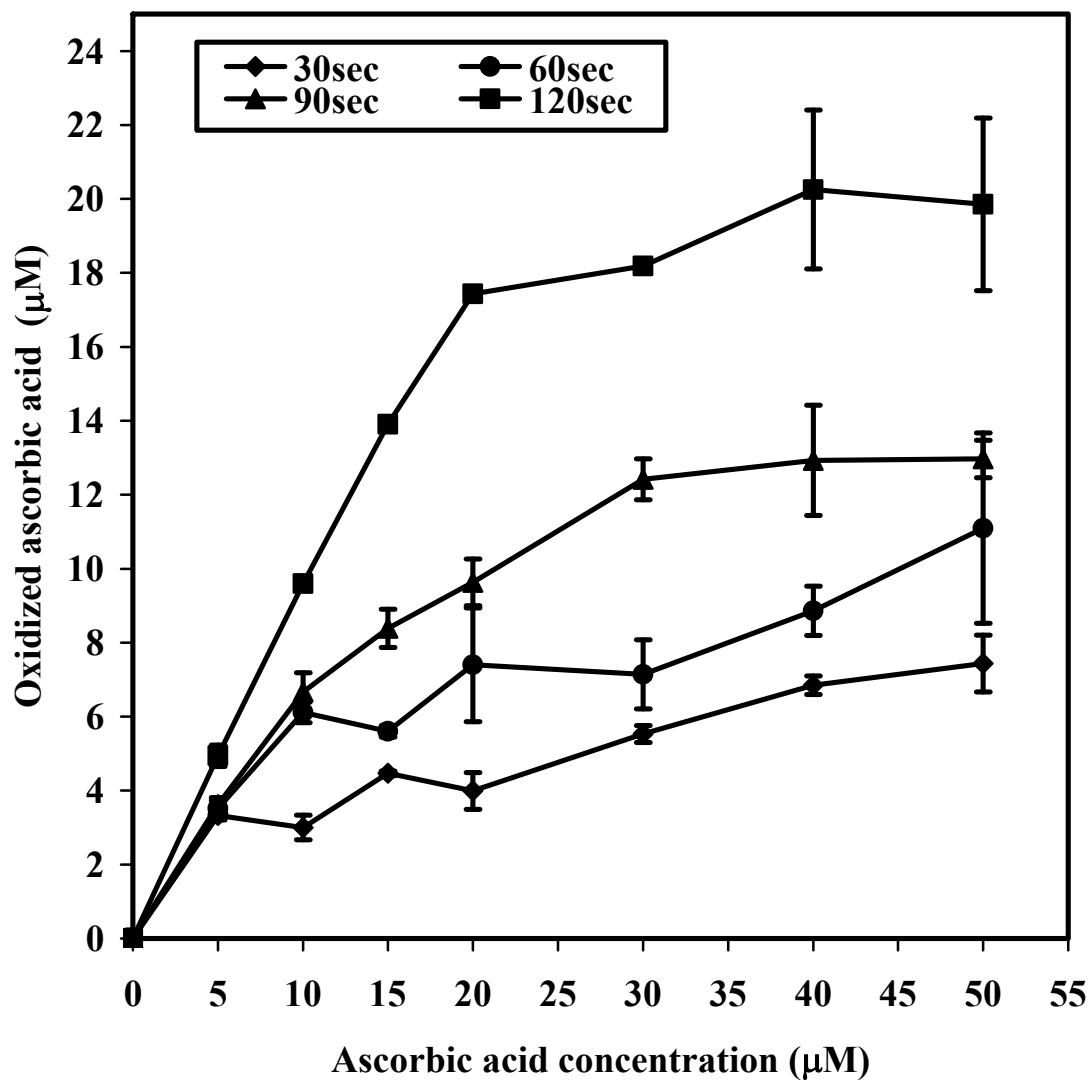
Figure 3.7 The effect of gamma irradiation in different concentration of ascorbic acid

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method. Experimental details are in Section 3.2.2. The data points were derived from three experiments (N=3, ± SD).

3.2.3 The effect of gamma irradiation of ascorbic acid oxidation under different gases

Ascorbate solutions were prepared daily. 20 μM ascorbate solutions were made up in 20 mM Chelex-treated phosphate buffer at pH 7.0. Samples were placed in an Oxford pipettor and irradiated at room temperature for 0 to 120 seconds (39.7 Gy/min). The sample solutions were pre-gassed by stirring under water-saturated argon or N_2O for 2 hours in an Oxford pipettor before irradiation in order to produce specific free radicals from water. During irradiation, the gas flow was maintained. Sample solutions in the presence of air were irradiated in Eppendorf tubes. At the each point of the irradiation time, 3×1 ml samples were taken out and incubated for 10 minutes at room temperature with 30 μl of dilute catalase (26 units) per ml solution to remove radiation generated hydrogen peroxide, before further experiments (Davies 2000). After addition of 2 M PCA, the ascorbic acid in each sample was analyzed using an absorbance maximum of λ_{max} at 244.5 nm on a spectrophotometer.

There results are showen in Figure 3.8. Without irradiation, there was no ascorbic acid oxidation under all three gases. After irradiation, the remaining amount of ascorbic acid dramatically decreased along with increasing irradiation time under all three gases. In the presence of air, the remaining ascorbic acid rapidly dropped from 20.0 μM to 6.5 μM after 60-second irradiation. Therefore, the level of ascorbic acid oxidation after irradiation reached about 67.6 %. Under N_2O, the remaining ascorbic acid was reduced from 20.0 μM to 9.9 μM after 60-second irradiation. The level of ascorbic acid oxidation after irradiation was 50.4 %.
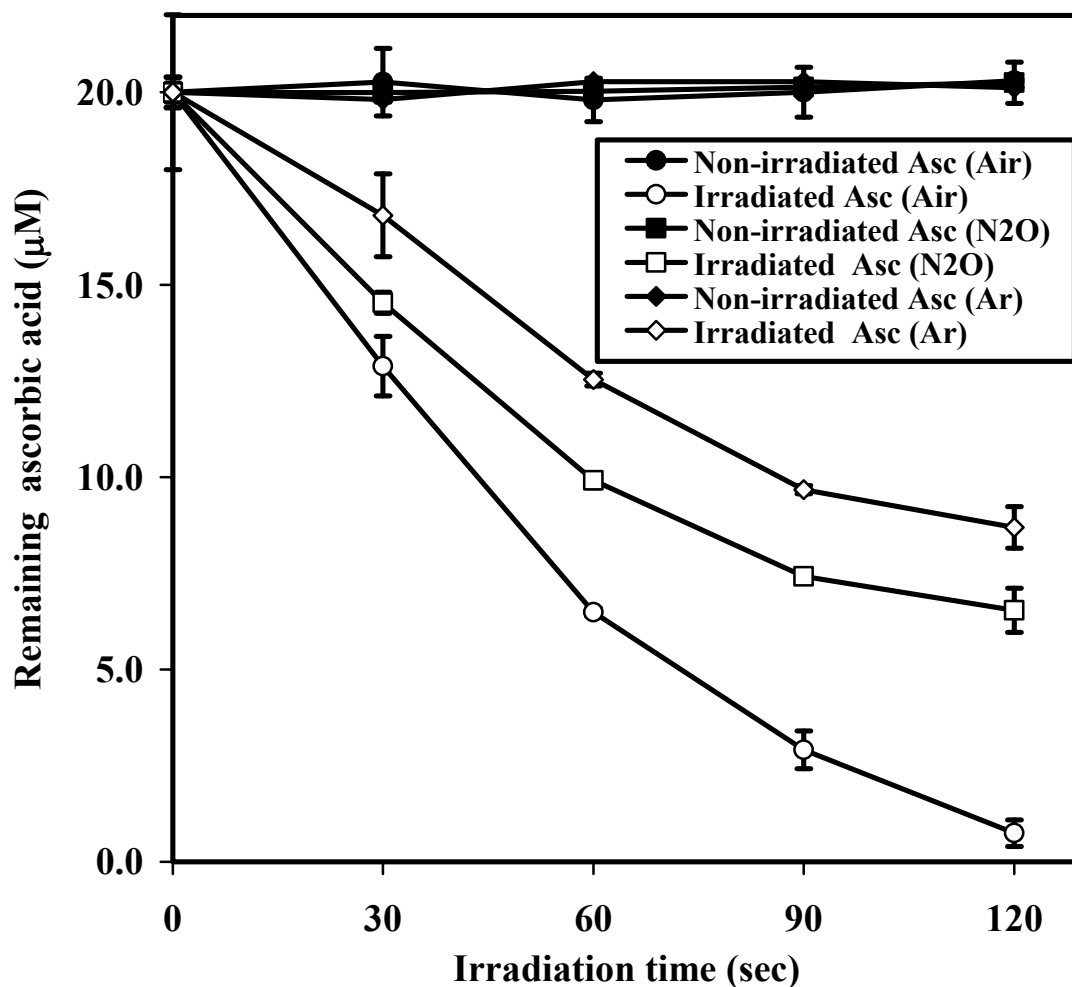
Figure 3.8 Effect of Co[60] γ-irradiation in ascorbic acid oxidation under different gases

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method. Experimental details are in Section 3.2.3. The data represent the means of six replicates, with standard deviations as indicated by bars.

In the presence of Argon, the amount of ascorbic acid decreased to 12.5 μM after 60-second irradiation, a loss of 37.3 %. To sum up, ascorbic acid was oxidized most efficiently in the presence of air which contains dioxygen.

Table 3.2 Comparison of the level of ascorbic acid oxidation after irradiation under air, argon and N_2O after 60-second irradiation

Atmosphere	Primary radicals	Oxidized ascorbic acid (μM)	G values
Argon	$HO^\bullet$	7.5	1.8
N_2O	$2 \times HO^\bullet$	10.1	2.5
Air	$HO^\bullet$, $O_2^{\bullet-}$	13.5	3.3

(G value listed is defined as number of oxidized ascorbic acid molecules per 100 eV of energy absorbed)

After 60-sec irradiation, 13.5 μM of ascorbic acid was oxidized by 11.1 μM $HO^\bullet$ and 13.2 μM $O_2^{\bullet-}$ in the presence of air. In the presence of N_2O, 10.1 μM ascorbic acid was oxidized by 22.2 μM $HO^\bullet$. In the presence of argon, 7.5 μM ascorbic acid was oxidized by 11.1 μM $HO^\bullet$. Under different gases, the irradiation systems yield specific radicals (Table 3.2). The rate constants for reaction of $HO^\bullet$ and $O_2^{\bullet-}$ have rate constants with ascorbic acid of about 10^{10} and 10^5 $M^{-1}s^{-1}$, respectively (Buettner 1988). The yield of $HO^\bullet$ is two times higher in the presence of N_2O than under argon. Under both N_2O and argon, $O_2^{\bullet-}$ is not produced. The reason why the yields of the different radicals generated do not correspond exactly to the amount of ascorbic acid oxidized is that the 20 μM ascorbic acid used was not sufficient for scavenging of all the radicals. This was done deliberately.

3.3 The oxidation of ascorbic acid by protein radicals

3.3.1 The effect gamma irradiation under different gases

Ascorbate solutions were prepared daily. Solutions of 20 μM ascorbate with 500 μM BSA or lysozyme were made up in 20 mM Chelex-treated phosphate buffer at pH 7.0. The reaction rate ratio of protein to ascorbate was between 100:1 and 111:1. The sample solutions were pre-gassed with argon or nitrous oxide for 2 hours in an oxford pipettor with stirring, with the gas flow maintained during the irradiation. Sample solutions in the presence of air were irradiated in eppendorf tubes. All solutions were irradiated at room temperature for 0 to 120 seconds.

At every 30 seconds of the irradiation time, 3×1 ml samples were taken out and incubated for 10 minutes at room temperature with 30 μl of dilute catalase (26 units) per ml solution to remove radiation-generated hydrogen peroxide (Davies 2000). The proteins were then precipitated with 2 M perchloric acid on ice for 20 min and the samples centrifuged at 16,500 rpm for 20 min at 4 $^{\circ}$C. The supernatant of each sample was analyzed for ascorbic acid content using an absorbance maximum of λ_{max} at 244.5 nm by a spectrophotometer.

3.3.2 The effect of BSA radicals in ascorbic acid oxidation under different gases

3.3.2.1 Oxidation under air

The initial 20 µM level of ascorbic acid was stable in non-irradiated sample solutions, both in the absence and in the presence of 500 µM BSA (Fig. 3.9). After irradiation, the remaining amount of ascorbic acid significantly declined in irradiated Asc and irradiated BSA + Asc groups. The presence of BSA in solution increased the oxidation of ascorbic acid. To sum up, after 60-sec irradiation, 35.8 % of ascorbic acid was oxidized in Irradiated Asc group. In Irradiated BSA + Asc group, 57.6 % of ascorbic acid was oxidized (Table 3.3). After 120-sec irradiation, almost 100 % of ascorbic acid was destroyed in irradiated BSA + Asc group. The results showed that the addition of BSA can enhance ascorbic acid oxidation.

Table 3.3 The percentage of oxidized ascorbic acid after irradiation in the presence of BSA under air (39.7 Gy/min)

Irradiation time (seconds)	% of oxidized ascorbic acid in total	
	Irradiated Asc	**Irradiated BSA + Asc**
60	35.8	57.5
120	87.9	99.6

121
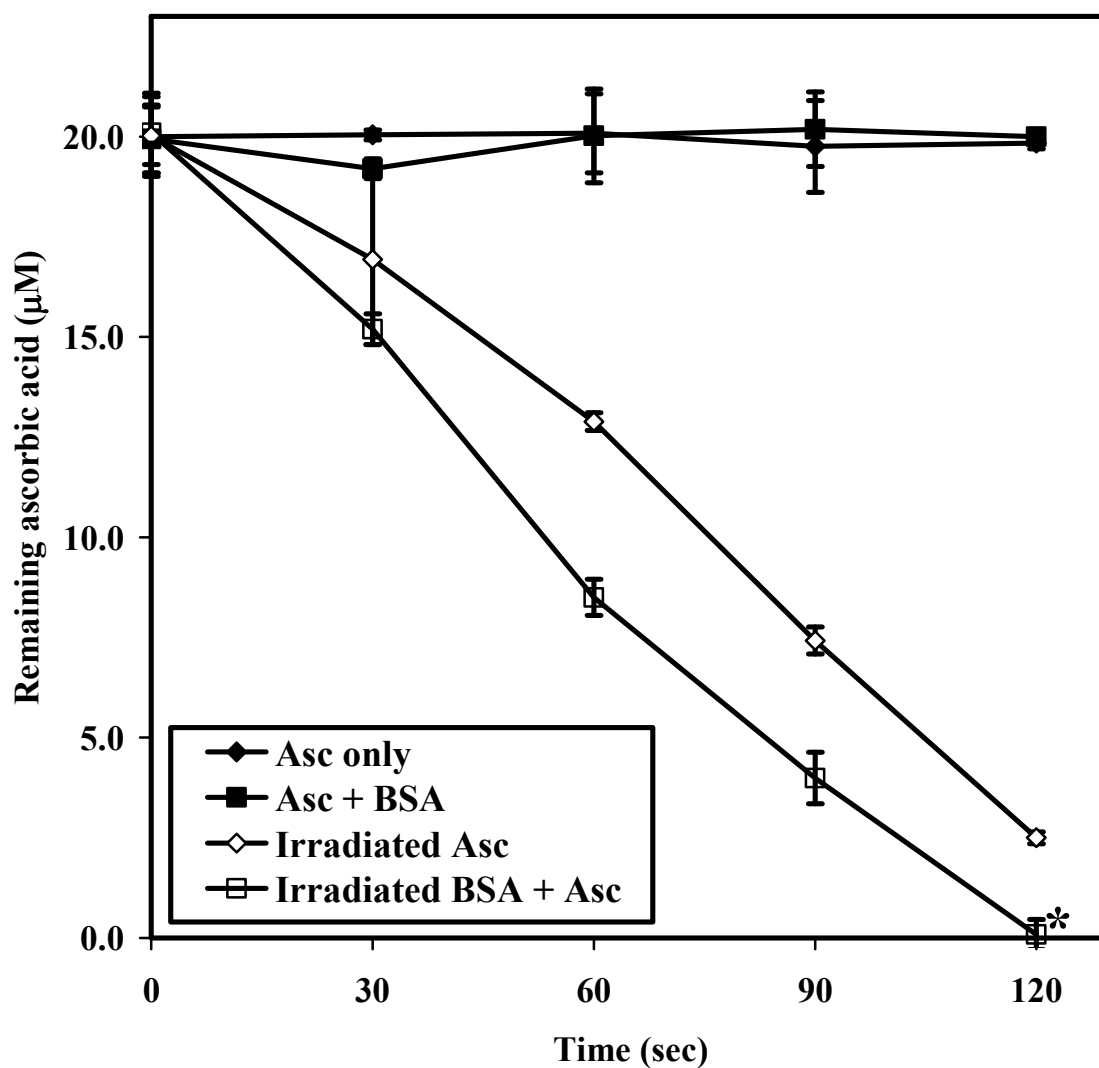
Figure 3.9 Effect of BSA radicals in ascorbic acid oxidation under air

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method. Experimental details are in Section 3.3.1. The data represent the means of triplicates, with standard deviations as indicated by bars. Asc: ascorbic acid; BSA: bovine serum albumin. *: $p < 0.05$.

3.3.2.2 Oxidation under N_2O and argon

Without irradiation, the level of ascorbic acid was maintained at 100 % under both argon and N_2O. With radiation (52.1 Gy/min), the remaining amount of ascorbic acid gradually decreased with increasing irradiation time in both Asc and BSA + Asc group. The amount of remaining ascorbic acid was significantly higher ($p<0.05$) in BSA-containing solution than in Asc only (Fig. 3.10). After 60-sec irradiation, in Asc only group, 55.5 % of ascorbic acid was oxidized under N_2O; 44.3 % of ascorbic acid was oxidized under Argon. The percentage of oxidized ascorbic acid was 25.4 % and 21.5 % under N_2O and Argon, respectively (Table 3.4). After 120-sec irradiation, the results were qualitatively similar. These results showed that ascorbic acid oxidation can be significantly limited by adding BSA in the presence of N_2O and Argon.

Table 3.4 The percentage of oxidized ascorbic acid after irradiation in the presence of BSA under N_2O and Argon (52.1 Gy/min)

Irradiation time (seconds)	% of oxidized ascorbic acid in total			
	Under N_2O		Under argon	
	Asc only	BSA + Asc	Asc only	BSA + Asc
60	55.5	25.4	44.3	21.5
120	74.1	43.3	52.9	36.0
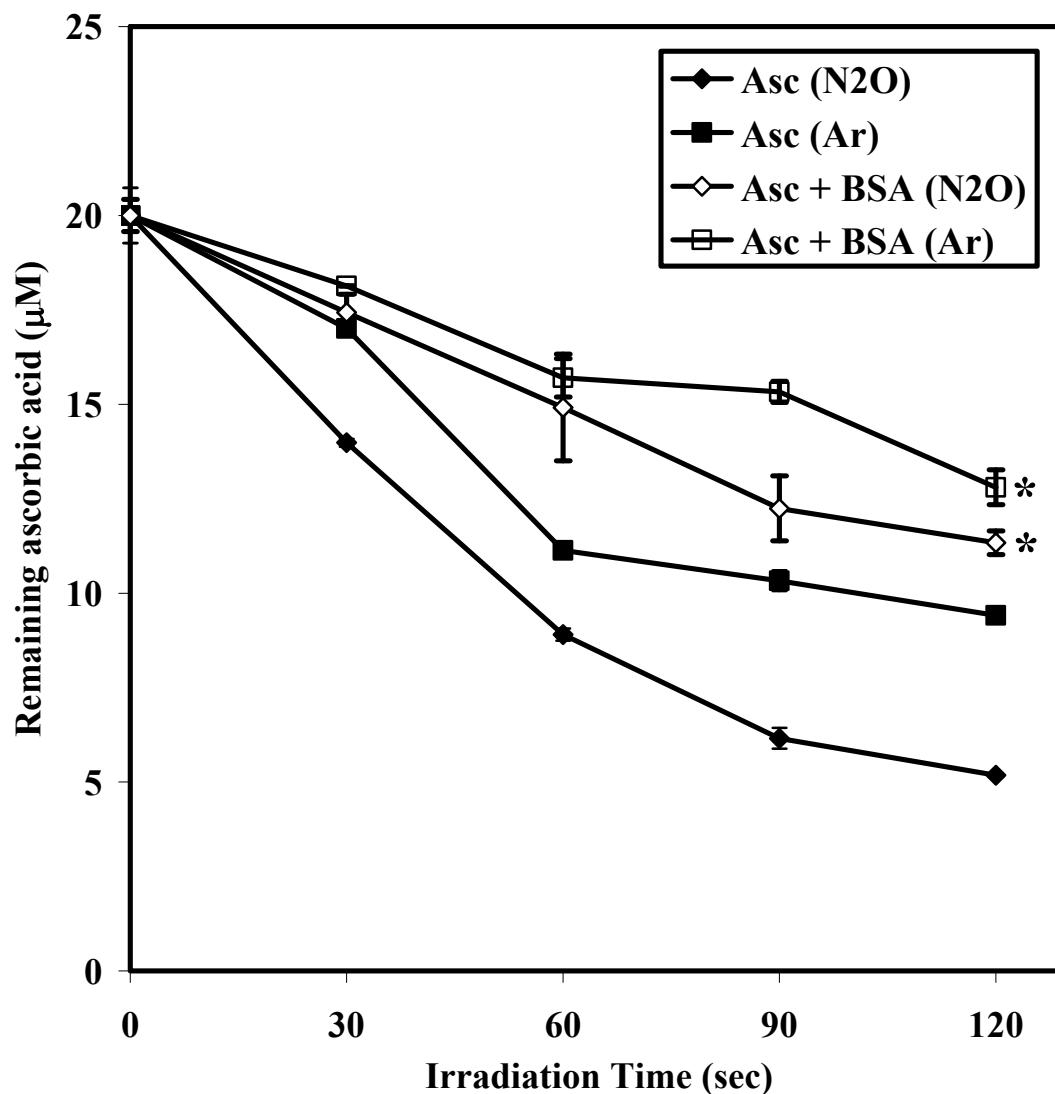
Figure 3.10 Effect of BSA in ascorbic acid oxidation under N₂O and Argon

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method. Experimental details are in Section 3.3.1. The data represent the means of triplicates, with standard deviations as indicated by bars. Asc: ascorbic acid; BSA: bovine serum albumin. . *: $p<0.05$.

3.3.2.3 Comparison of the effects of BSA radicals in ascorbic acid oxidation under different gases

Results in Table 3.5 show the combined effects of BSA radicals on ascorbic acid oxidation under three gases. Without irradiation, the amount of ascorbic acid was steady for the 120 seconds incubation in all three gas conditions (only the results under two gases are shown). With irradiation, the ascorbic acid concentration dropped with increasing irradiation time under all three gases, especially in the presence of air. In BSA-containing group, the sequence of the ability to oxidize ascorbic acid with radiation was under Air > N_2O > argon. The reaction rate ratio of BSA and ascorbic acid in the sample solutions was about 100 to 1. All of the HO$^\bullet$ radicals generated by gamma irradiation would react with BSA (Gebicki and Gebicki 1993) to produce BSA radicals, such as BSA$^\bullet$ and BSAOO$^\bullet$ in the presence of air. In addition, BSAOOH would be produced, which slowly oxidizes ascorbic acid (Simpson, Narita et al. 1992). Table 3.5 shows the different radicals produced under different gases.

The level of ascorbic acid oxidation can be enhanced by the addition of BSA in the presence of air, but not under N_2O or argon. Almost all ascorbic acid in solution was oxidized by BSA$^\bullet$ and BSAOO$^\bullet$ radicals in 120s in the presence of air, the G value was 2.4. The BSA$^\bullet$ and BSAOO$^\bullet$ radicals produced more oxidized ascorbic acid than HO$^\bullet$, because the 500 µM BSA scavenged all of primary radicals, while the 20 µM ascorbic acid did not (Fig. 3.9). The level of ascorbic acid oxidation was slightly lower in the presence of BSA than in Asc only solution under N_2O. The results under argon were similar. The amount of oxidized ascorbic acid was 1.5 fold greater in the Asc only group than BSA/Asc group

under argon. These results illustrated the high efficiency of the HO$^\bullet$ radicals in oxidizing ascorbic acid in comparison with the BSA radical in these systems, suggesting that, when no BSAOO$^\bullet$ was generated, the C-centred protein radicals were relatively inefficient ascorbate oxidants. However, these results do not allow an estimate of the contribution of superoxide radicals to the loss of ascorbate, so that conclusions on the relative importance of the different radicals generated in ascorbate oxidation must be treated with caution.

Table 3.5 The amount of ascorbic acid oxidized by different radicals under air, argon and N$_2$O after 120 second irradiation (BSA)

Gas	BSA	Primary radicals	Secondary radicals	Oxidized ascorbic acid (µM)	G value
Argon	-	HO$^\bullet$	-	10.6	1.3
Argon	+	HO$^\bullet$	BSA$^\bullet$	7.2	0.9
N$_2$O	-	2 × HO$^\bullet$	-	14.8	1.8
N$_2$O	+	2 × HO$^\bullet$	2 × BSA$^\bullet$	8.7	1.1
Air	-	HO$^\bullet$, O$_2^{\bullet-}$	-	17.5	2.1
Air	+	HO$^\bullet$, O$_2^{\bullet-}$	BSA$^\bullet$, BSAOO$^\bullet$, O$_2^{\bullet-}$	19.9	2.4

(G value listed defined as number of ascorbate molecules oxidized per 100eV of energy absorbed)

3.3.3 The effect of lysozyme radicals on ascorbic acid oxidation under different gases

The experimental details in this study were similar to those for BSA (Section 3.3.2).

3.3.3.1 Oxidation under air

The amount of ascorbic acid remained unchanged in un-irradiated sample solutions. Lysozyme (LZ) alone did not induce ascorbic acid oxidation (Fig. 3.11). After irradiation, the amount of ascorbic acid gradually reduced with increasing irradiation time in both irradiated Asc and irradiated LZ + Asc group. The presence of 500 μM LZ in solution made no difference to the levels of ascorbic acid remaining. The percentage of oxidized ascorbic acid was roughly proportional to the length of irradiation (Table 3.6). After 120-sec irradiation, almost 90 % of ascorbic acid was destroyed in the irradiated BSA + Asc group. The results showed that under air, BSA radicals were better oxidants of ascorbate than lysozyme radicals.

Table 3.6 The percentage of oxidized ascorbic acid after irradiation in the presence of LZ under air (37.9 Gy/min)

Irradiation time (seconds)	% of oxidized ascorbic acid in total	
	Irradiated Asc	Irradiated LZ + Asc
60	40.0	39.9
120	93.8	88.7
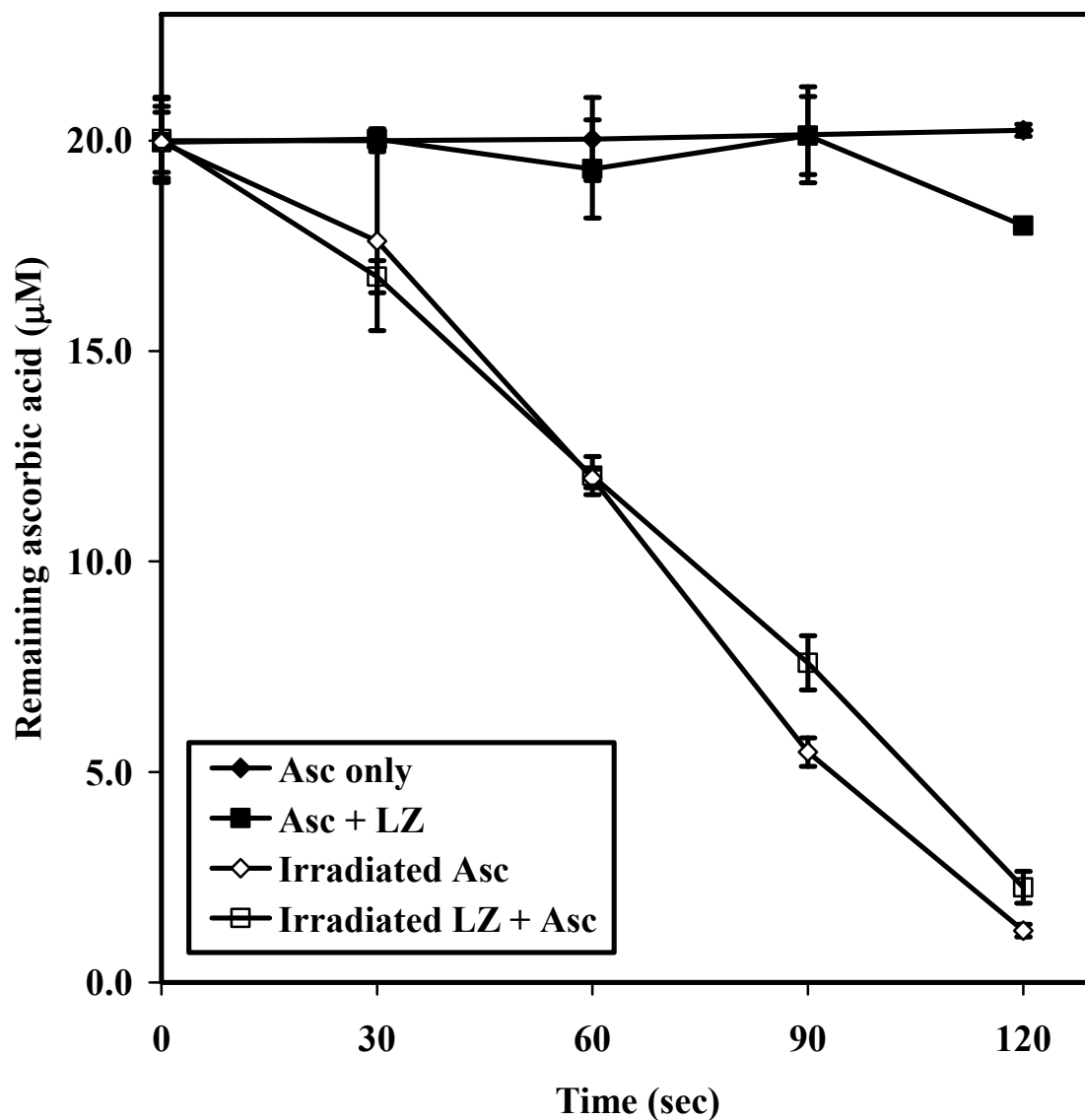
Figure 3.11 Effect of LZ radicals in ascorbic acid oxidation under air

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method. Experimental details are in Section 3.3.1. The data represent the means of triplicates, with standard deviations as indicated by bars. Asc: ascorbic acid; LZ: Lysozyme.

3.3.3.2 The effect of lysozyme radicals on ascorbic acid oxidation under N_2O and argon

Without irradiation, the level of ascorbic acid remained at 100% under both argon and N_2O in all groups. After radiation (52.1Gy/min), the amount of ascorbic acid gradually reduced with increasing irradiation time in both Asc and LZ + Asc groups under the two atomspheres. The amount of remaining ascorbic acid was significantly higher ($p<0.05$) in LZ-containing solution than in the Asc one under Argon (Fig. 3.12). After 60-sec irradiation, the percentage of oxidized ascorbic acid in the irradiated solution containing lysozyme was 30.8 % and 19.0 % under N_2O and Argon, respectively. This was significantly lower than in the Asc only solutions (Table 3.7). After 120-sec irradiation, the results were similar with more ascorbic acid lost in each group. To sum up, ascorbic acid oxidation was significantly reduced by the LZ radicals under N_2O or Argon by comparison with the results under air (Fig. 3.11).

Table 3.7 The percentage of oxidized ascorbic acid after irradiation in the presence of LZ under N_2O and Argon (52.1 Gy/min)

| Irradiation time (seconds) | % of oxidized ascorbic acid in total | | | |
| | Under N_2O | | Under argon | |
	Asc only	LZ + Asc	Asc only	LZ + Asc
60	55.5	30.8	44.3	19.0
120	74.1	38.5	52.9	21.8

Figure 3.12 Effect of LZ in ascorbic acid oxidation under N$_2$O and Argon

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method. Experimental details are in Section 3.3.1. The data represent the means of triplicates, with standard deviations as indicated by bars. Asc: ascorbic acid; and LZ: Lysozyme. *: $p<0.05$.

3.3.3.3 Comparison of the effects of LZ radicals on ascorbic acid oxidation under different gases

The combined results in Figure 3.11 and 3.12 show the effect of LZ radicals on ascorbic acid oxidation under three gases. Without irradiation, the amount of ascorbic acid was steady for the 120 seconds incubation under all three gases (only the results under two atmospheres are shown). With irradiation, the ascorbic acid concentration dropped with increasing irradiation time under all three gases, especially in the presence of air. In LZ-containing group, the sequence of amounts of ascorbic acid oxidized under three gases by irradiation was air > N_2O > argon.

The ratio of reaction rates of $HO^\bullet$ radicals with LZ and ascorbate in the sample solutions was 111 to 1. The 99 % of $HO^\bullet$ radicals produced by gamma irradiation generated LZ protein radicals, $LZ^\bullet$ and $LZOO^\bullet$ in the presence of air. These radicals oxidized ascorbic acid, but the LZOOH also generated reacted too slowly to result in significant loss of ascorbate in these experiments. However, in the presence of air, the superoxide radicals are also able to oxidize ascorbate with a rate constant of $5 \times 10^4 \, M^{-1}s^{-1}$ (Cabelli, Comstock et al. 1983). Table 3.8 lists the radicals generated in these experiments.

The level of ascorbic acid oxidation was reduced by the addition of LZ in the presence of N_2O or argon, but only slightly under air. The decrease was highly significant under N_2O or argon. These results show that lysozyme radicals were less effective oxidants than $HO^\bullet$, especially as not all of the $HO^\bullet$ were scavenged in the absence of the proteins.   In the presence of air, the levels of oxidized ascorbic acid were similar in the Asc only group and

in the LZ-containing group. Here, superoxide radicals clearly made a significant contribution to ascorbate oxidation.

Table 3.8 The amount of ascorbic acid oxidized by different radicals under air, argon and N_2O after 120 second irradiation (LZ)

Gases	BSA	Primary radicals	Secondary radicals	Oxidized ascorbate (μM)	G value
Argon	-	$HO^\bullet$	-	10.4	1.3
Argon	+	$HO^\bullet$	$LZ^\bullet$	*4.4	0.5
N_2O	-	$2 \times HO^\bullet$	-	14.8	1.8
N_2O	+	$2 \times HO^\bullet$	$2 \times LZ^\bullet$	*7.7	0.8
Air	-	$HO^\bullet$, $O_2^{\bullet-}$	-	17.5	2.1
Air	+	$HO^\bullet$, $O_2^{\bullet-}$	$LZ^\bullet$, $LZOO^\bullet$, $O_2^{\bullet-}$	17.8	2.2

(G value listed defined as number of ascorbate molecules oxidized per 100eV of energy absorbed) *: $p < 0.05$.

3.3.4 The effect of superoxide dismutase on radical-induced ascorbic acid oxidation

In order to determine the contribution of the $O_2^{\bullet-}$ radicals to the oxidation of ascorbate in the presence of dioxygen, measurement of ascorbate oxidation under air were performed in the presence of 50 units of superoxide dismutase (SOD) in every ml of the irradiated sample.

Solutions of 20 μM ascorbic acid with or without 500 μM BSA were made up in 20 mM Chelex-treated phosphate buffer at pH 7.0. The reaction rate ratio of protein to ascorbic acid was 100:1. Samples were placed in Eppendorf tubes and irradiated at room temperature for 0 to 120 seconds. Under air-saturated condition, superoxide anion ($O_2^{\bullet-}$) was formed. Before irradiation, 50unit/ml SOD was added into sample solutions. The $O_2^{\bullet-}$ generated was dismutated enzymatically through action of superoxide dismutase (Nappi 1997). At the end of the irradiation time, each sample was incubated for 10 minutes at room temperature with catalase (26 units). Proteins were then precipitated with 2M perchloric acid (PCA) on ice for 20 min and then centrifuged at 16,500 rpm for 20 min at 4 ^{o}C. The supernatant of each sample was analyzed for ascorbic acid using an absorbance maximum of λ_{max} at 244.5 nm by a spectrophotometer.

The high levels of oxidation of ascorbic acid by protein radicals in the presence of dioxygen compared to its absence (Fig. 3.13 and Table 3.9) suggested that superoxide radicals made a significant contribution to the loss of ascorbate. Without SOD treatment, the amount of oxidized ascorbic acid was about twice as high than in the presence of SOD.

The HO$^\bullet$ was slightly more effective than BSA$^\bullet$, and BSA$^\bullet$/BSAOO$^\bullet$ were slightly more efficient oxidants of ascorbic acid than superoxide anion radicals. In general terms, the amount of ascorbic acid oxidized by HO$^\bullet$ and BSA$^\bullet$ radicals were approximately equal and it about doubled in the presence of dioxygen.

Table 3.9 The level of ascorbic acid oxidation by various free radicals and the effect of superoxide dismutase

Substrate	SOD	Oxidized ascorbic acid (μM)	Primary radicals	Effective radicals
Asc	-	18.5	HO$^\bullet$, O$_2^{\bullet-}$	HO$^\bullet$, O$_2^{\bullet-}$
Asc	+	*10.8	HO$^\bullet$	HO$^\bullet$
Asc + BSA	-	19.9	HO$^\bullet$, O$_2^{\bullet-}$	BSA$^\bullet$, BSAOO$^\bullet$, O$_2^{\bullet-}$
Asc + BSA	+	* 9.4	HO$^\bullet$	BSA$^\bullet$, BSAOO$^\bullet$

Asc: ascorbic acid; -: without SOD; +: with SOD; and BSA: bovine serum albumin. *: $p < 0.05$. Effective radicals are those responsible to oxidation of ascorbic acid. Irradiation time was 120 s.
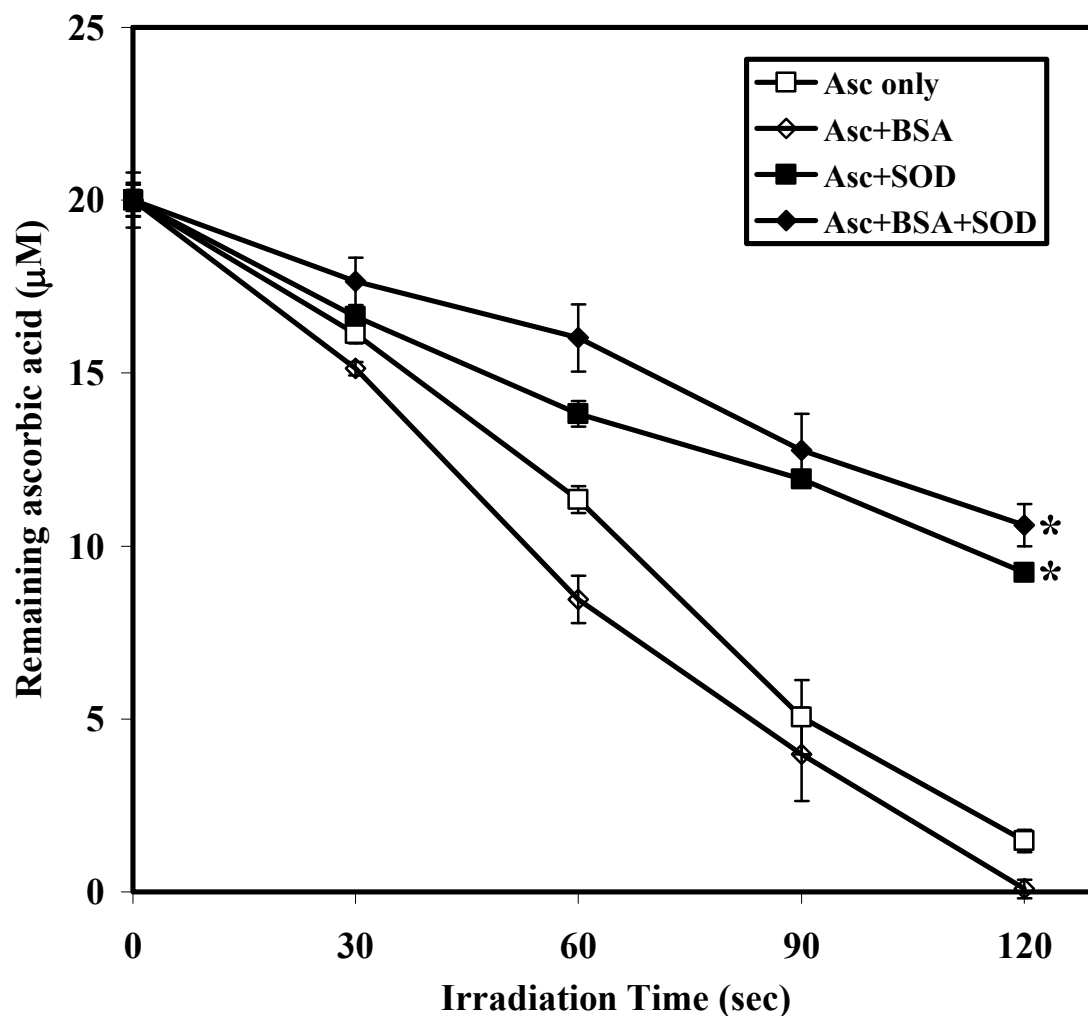
Figure 3.13 Effect of superoxide dismutase in irradiated ascorbic acid and BSA under air

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method. Experimental details are in Section 3.3.4. The data points were derived from 2 experiments with error bars (N=3, ± SD). Asc: Ascorbic acid; SOD: superoxide dismutase; and BSA: Bovine serum albumin.

3.3.5 The effect of different protein radicals on ascorbic acid oxidation

Five proteins: BSA, lysozyme, human serum albumin, α-casein, and chymotrypsin were used in this experiment. 20 μM ascorbic acid with or without protein were made up in 20 mM phosphate buffer at pH 7.0. The reaction rate ratio of protein to ascorbic acid was between 23:1 and 170:1. The sample solutions were then irradiated at room temperature for 0 or 90 seconds in Eppendorf tubes. At the end of the irradiation time, each sample was incubated with 26 units of catalase for 10 minutes at room temperature. Proteins were then precipitated with 2 M PCA on ice for 20 min and then centrifuged at 16,500 rpm for 20 min at 4 ^{o}C. The supernatant of each sample was analyzed for ascorbic acid using an absorbance maximum of λ_{max} at 244.5 nm by a spectrophotometer.

Between 96 % and 99 % of HO$^{\bullet}$ radicals generated form gamma irradiation reacted with the proteins first and then produced the protein radicals, as listed in Table 3.10. These specific protein radicals together with the O$_2^{\bullet-}$ oxidized ascorbic acid (Fig. 3.14) the contribution of O$_2^{\bullet-}$ to the total amount of ascorbate oxidized can be roughly estimated by assuming that, by analogy with the experiments with BSA (Fig. 3.13) about 8.0 μM ascorbate was also oxidized by O$_2^{\bullet-}$ in the presence of the other proteins. If this assumption is correct, HSA radicals were the most effective oxidants and chymotrypsin radicals the less effective. However, such estimate does not take into account the possible reaction between superoxide and protein radicals. This would result in loss of potential oxidants of ascorbate. The influence of the superoxide-protein radical reactions on the overall amounts of ascorbate oxidized cannot be calculated, because the rate constants of such reactions have not been measured.

Table 3.10 Reaction rate ratio of hydroxyl radicals with ascorbic acid and different proteins and the related radicals

Substrate	Attacking Radical [c]	Rate constant $(dm^3m^{-1}s^{-1})$ [d]	Reaction rate ratio to Asc⁻	Radical generated [e]
Ascorbic acid	HO•	1.1×10^{10} [a]	1	Asc•
Bovine serum albumin	HO•	2.5×10^{10} [a]	100	BSA•
Lysozyme	HO•	4.9×10^{10} [a]	111	LZ•
Human serum albumin	HO•	7.5×10^{10} [a]	170	HSA•
α-casein	HO•	$\sim 10^{10}$ [b]	23	Cas•
Chymotrypsin	HO•	3.7×10^{10} [a]	84	Chy•

Reference: a: (Buxton, Greenstock et al. 1988); b:(Halliwell and Gutteridge 1999); c: superoxide radicals were also generated with HO• (not shown); d: rate constant for reaction of HO• and protein; e: except for line 1; other radicals shown were generated on the proteins.

Without irradiation, there was no protein radical production, and the amount of ascorbic acid remained at 100 % of the original concentration in the different protein solutions. To sum up, all five protein radicals can cause ascorbic acid oxidation with different effectiveness.

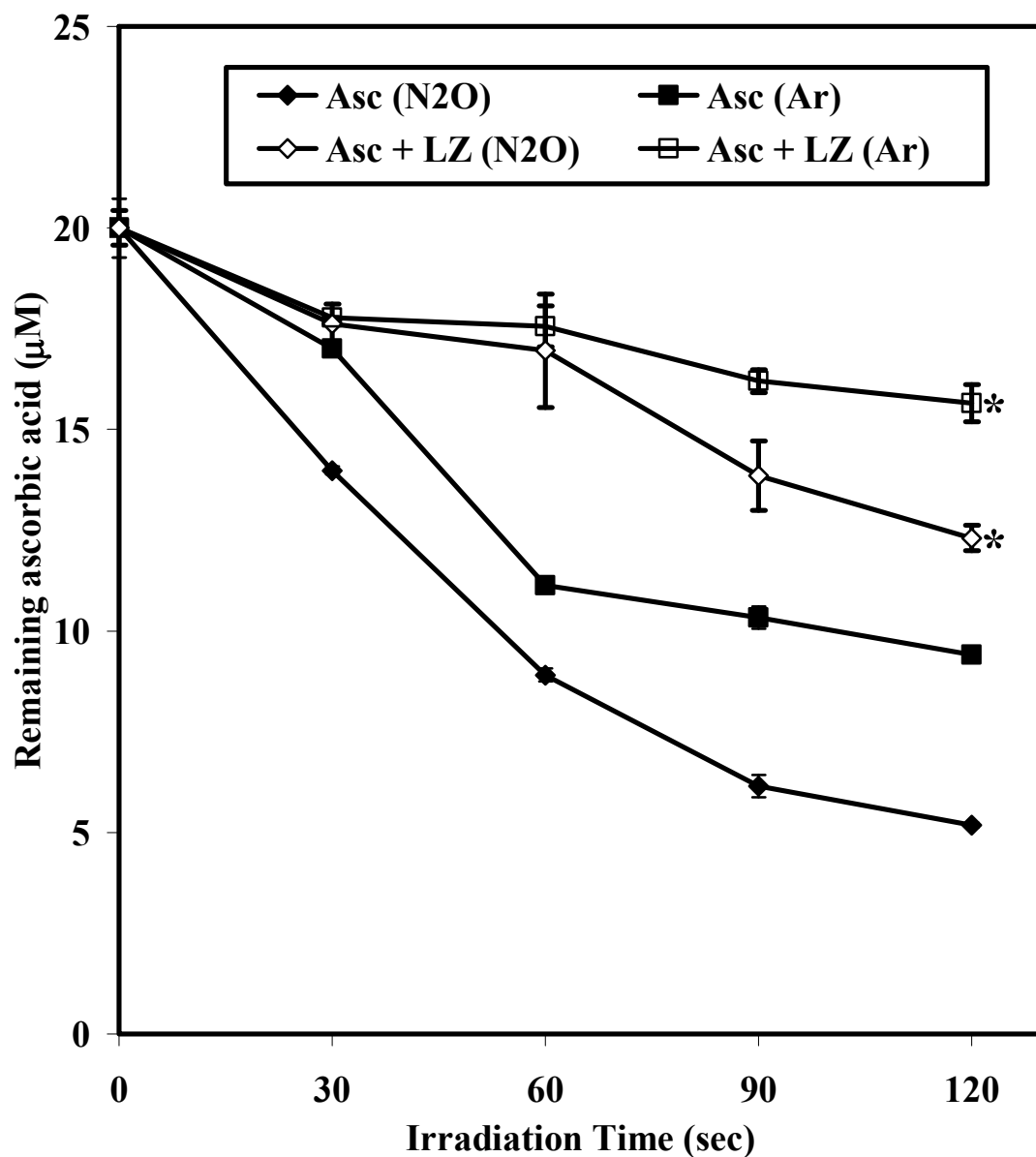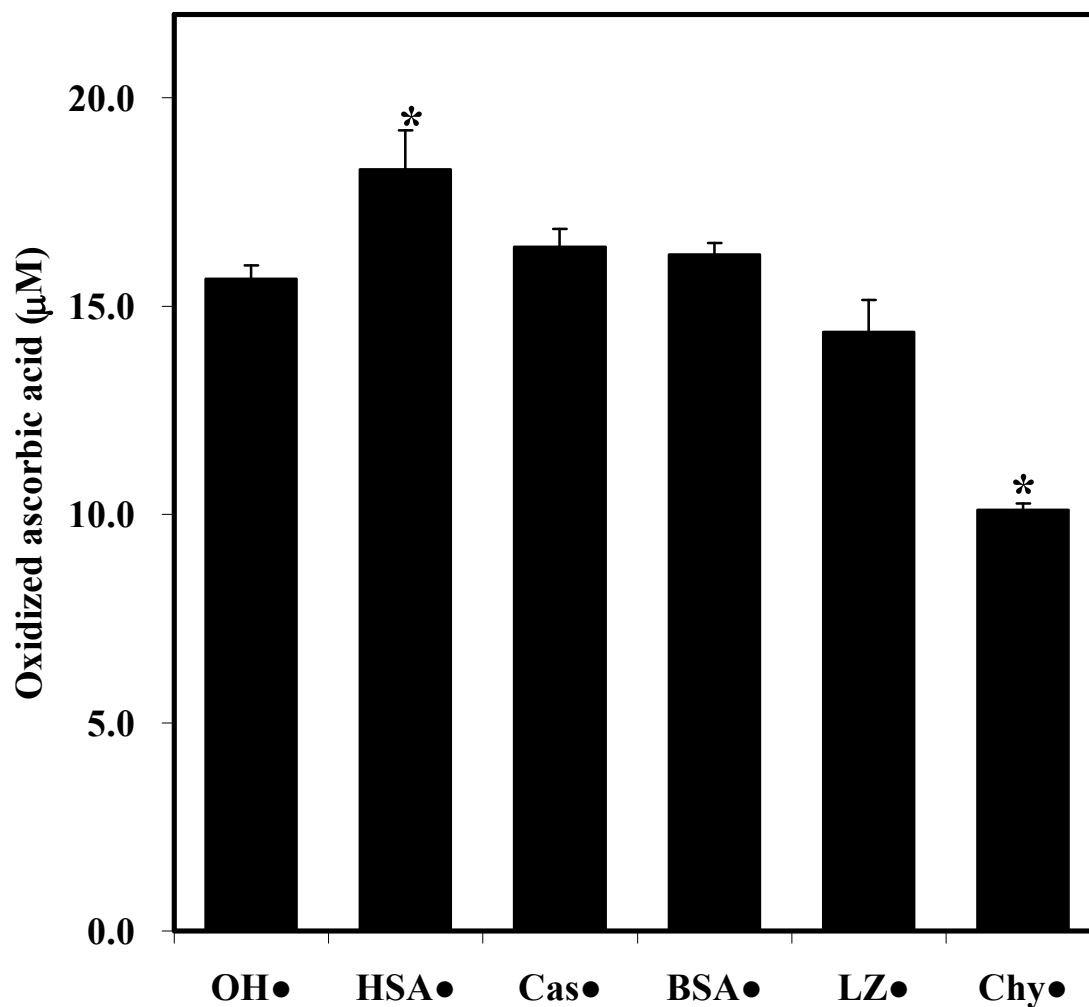**Figure 3.14 The effect of different protein radicals in ascorbic acid oxidation**

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method. Experimental details are in Section 3.3.5. Irradiation time was 90s. The data points were derived from 4 experiments with error bars (N=4, ± SD). HSA: Human serum albumin; Cas: α-casein; BSA: Bovine serum albumin; LZ: lysozyme; and Chy: Chymotrypsin. *: $p<0.05$. The bars include contribution from $O_2^{\bullet-}$ which may be as high as ≈ 8.0 μM of ascorbic acid oxidized.

3.4 The effect of hydroxyl and superoxide anion radicals on amino acid radicals-induced ascorbic acid oxidation

3.4.1 Determination of amino acid concentration used in experiments

In order to make the hydroxyl and superoxide anion radicals in solution react with amino acids first, the reaction rate ratio of amino acid (AA) to ascorbic acid was set as at least 20/1. Under this condition, 95 % of HO$^{\bullet}$ would react with amino acids producing amino acid radicals which could further oxidize the ascorbic acid. The concentrations of 17 different amino acids used are listed in Table 3.11.

Table 3.11 Reaction rate ratio of hydroxyl radicals with ascorbic acid and different amino acids and the related radicals (Halliwell and Gutteridge 1999). * Amino acid concentration needed to ensure reaction with 95 % of hydroxyl radicals generated.

Amino acids	k(OH) × 10^{-7} (dm^{3}m^{-1}s^{-1})[a]	Desired conc. (mM)*	Used conc. (mM)
Ala	7.7	23.38	50
Val	76	2.37	10
Ile	180	1.00	10
Leu	170	1.06	10
Met	830	0.22	10
Pro	48	3.75	10
Gly	1.7	105.88	120
Ser	32	5.63	10
Thr	51	3.53	10
Glu	23	7.83	10
Asp	7.5	24.00	40
Cys	3.4	52.94	100
Asn	4.9	36.73	50
Gln	54	3.33	10
His	500	0.36	10
Arg	350	0.51	10
Lys	35	5.14	10

3.4.2 The effect of different amino acid radicals on ascorbic acid oxidation

A selection of 17 amino acids at 10-120 mM was irradiated with 20 μM ascorbic acid by gamma radiation for 0 and 60 seconds (0 and 39.7 Gy) in the phosphate pH 7.0 buffer system. The aromatic amino acids were not studied because their absorbance interferes with the ascorbic acid assay. The reaction rate ratio of hydroxyl radicals with amino acid to ascorbic acid was above 20:1. Before irradiation, 50 unit/ml SOD was added to duplicate sample solutions. After irradiation, the ascorbic acid absorbance was read directly at 255 nm, the maximum absorption peak of L-ascorbic acid in neutral solution, in a spectrophotometer.

During irradiation, AA$^\bullet$ and AAOO$^\bullet$ radicals were produced, for instance, Ala $^\bullet$ and AlaOO $^\bullet$. Superoxide anion radicals which oxidized ascorbate (Cabelli, Comstock et al. 1983) were removed same samples by the presence of 50 units of SOD during the irradiation.

Figure 3.15 shows that the level of ascorbic acid oxidation was significantly decreased by SOD treatment in samples of ascorbic acid only and in samples containing Ala, Pro, Gly, Ser, Thr, Glu, Asn, and His ($p<0.01$). In the absence of SOD, amino acid radicals produced lower amounts of oxidized ascorbic acid than hydroxyl radicals, especially Cys, Met, Asp, and Gln. Ascorbate was most readily oxidized by radicals from Val, Ile, and Leu (more than 75 %) (Table 3.12). Gly, Ser, Thr, Asn radicals were less able to oxidize ascorbate.

Ascorbate oxidation was significantly reduced in the presence of SOD, confirming that under these conditions, superoxide was an effective oxidant. The presence of cysteine also resulted in complete inhibition of ascorbate oxidation by $O_2^{\bullet-}$ which was evident with all the other amino acids. There is insufficient information to allow calculations of the contributions of superoxide and amino acid radicals to ascorbate oxidation. Moreover, the results obtained in the presence of SOD indicate that all of the amino acid radicals tested had the ability to oxidize ascorbate with different efficiency, except Cys.

Table 3.12 The percentage of ascorbate oxidized by different amino acid radicals under air

% of ascorbate oxidized by AA radicals	Amino acid
0-25	Cys, Gly, Ser, Thr, Asn
25-50	Ala, Met, His
50-75	Pro, Glu, Asp, Gln, Arg, Lys
75-100	Val, Ile, Leu
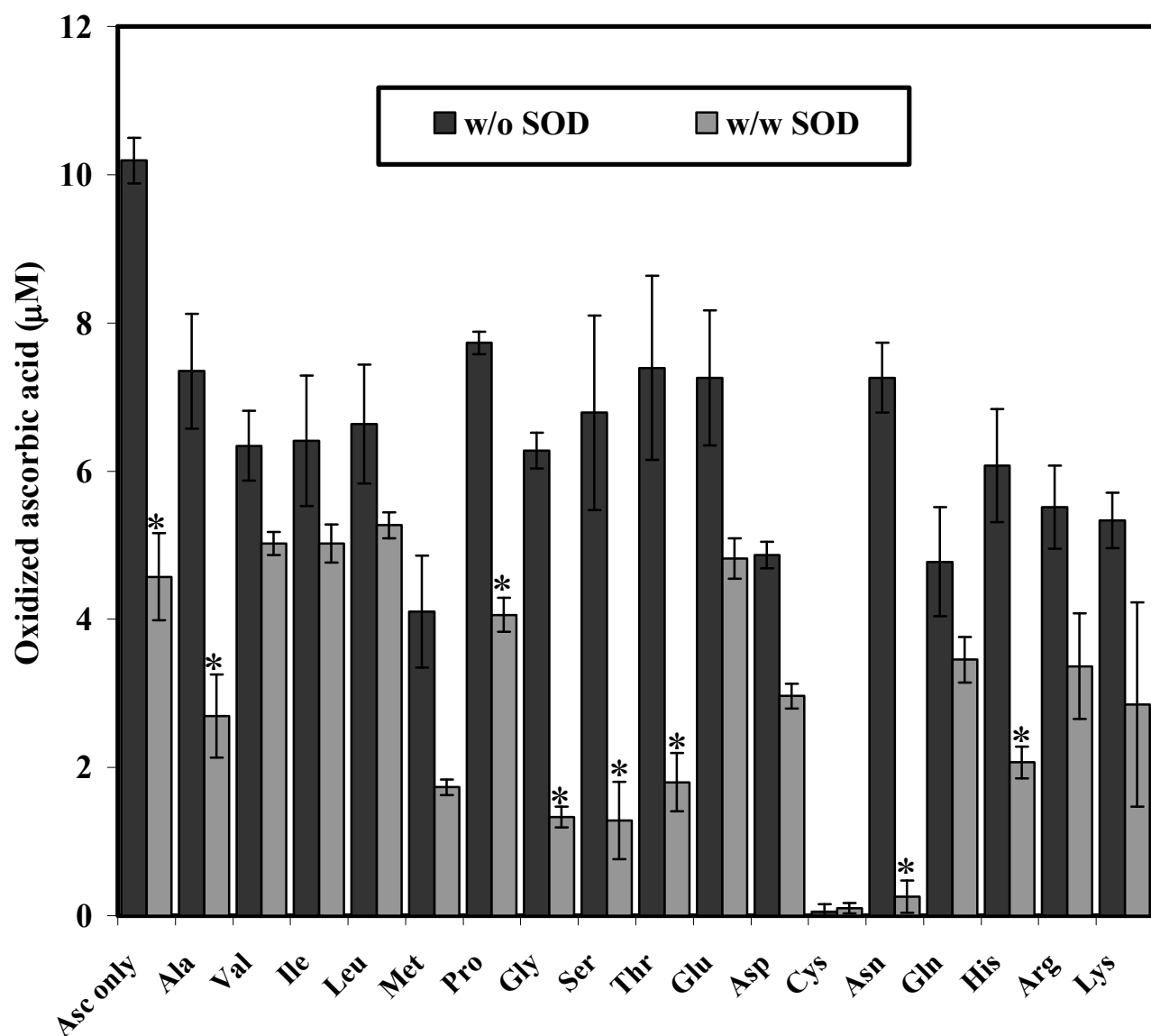
Figure 3.15 Effect of amino acid radicals in ascorbic acid oxidation

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method. Experimental details are in Section 3.4.2. The data represent the means of three replicates, with standard deviations as indicated by bars. Asc: ascorbic acid only; Ala; Alanine; Ile: Isoleucine; Gly: Glycine; Cys: Cysteine; Arg: Arginine; Asp: Aspartic acid; Lys: Lysine; Glu: Glutamine; Asn: Asparagine; Ser: Serine; Gln: Glutamic acid; Thr: Threonine; Leu: Leucine; Met: Methionine; Pro: Proline; and His: Histidine. w/w SOD: with SOD. w/o: without SOD. * Indicates statistically significant difference between SOD and non-SOD treatment samples at $p<0.01$ level.

The results shown in Figure 3.15 reflect a complicated pattern of competing reactions.

Over 95 % of the HO$^{\bullet}$ reacted with the amino acid present:

$$HO^{\bullet} + AAH \rightarrow H_2O + AA^{\bullet} \tag{3.6}$$

The C-centred radicals can react with O_2 ($k_* = 2 \times 10^9$ $M^{-1}s^{-1}$) (Von Sonntag 1987)

$$AA^{\bullet} + O_2 \rightarrow AAOO^{\bullet} \tag{3.7}$$

or with ascorbate:

$$AA^{\bullet} + AscH^- \rightarrow AAH + A^{\bullet -} \tag{3.8}$$

The only known rate constant of reaction 3.8 is for Trp radicals reported at 10^8 $M^{-1}s^{-1}$ (Hoey and Butler 1984). Rate constant of reaction 3.7 is likely to be about 10^9 $M^{-1}s^{-1}$, so that the formation of AAOO$^{\bullet}$ will predominate in O_2-saturated solution and ascorbate will be oxidized mainly in reaction 3.9.

$$AAOO^{\bullet} + AscH^- \rightarrow AAOOH + A^{\bullet -} \tag{3.9}$$
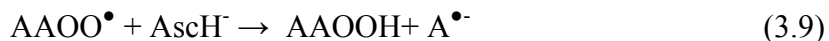
Occurrence of reaction should result in reaction 3.9 and direct oxidation of ascorbate by superoxide equal amounts of ascorbate oxidized by AA radicals, if their rate constants are similar. However, as shown by the gray bars in Figure 3.15, cross-reaction between the superoxide radicals and reaction with amino acid radicals resulted in differences in overall effectiveness of the AA radicals.

In the absence of SOD, formation of $O_2^{\bullet -}$ introduced additional possible cross reaction. This was not evident in the case of Cys, the only amino acid reacting with superoxide at significant rate:

$$Cys + O_2^{\bullet-} \rightarrow \text{ product} \qquad\qquad\qquad (3.10)$$

Evidently, the $O_2^{\bullet-}$ was effectively removed by the reactively high Cys concentration (Bielski, Cabell et al. 1985) and the S-centred Cys radical formed reacted with O_2 to generate the unreactive $CysOO^{\bullet}$ (Schoneich, Dillinger et al. 1992).

3.5 Effect of protein on the oxidation of ascorbic acid by superoxide radicals

3.5.1 Superoxide anion radical generation

The sodium formate (HCOONa) water solution produces reducing radicals, $O_2^{\bullet-}$ and $CO_2^{\bullet-}$, after gamma irradiation in the presence of O_2. The carbon dioxide radical can convert O_2 to $O_2^{\bullet-}$. Without O_2, only carbon dioxide radicals would be produced (Halliwell and Gutteridge 1999).

$$e_{aq}^- + O_2 \rightarrow O_2^{\bullet-} \qquad\qquad\qquad (3.11)$$

$$H^{\bullet} + HCOO^- \rightarrow H_2 + CO_2^{\bullet-} \qquad\qquad\qquad (3.12)$$

$$HO^{\bullet} + HCOO^- \rightarrow H_2O + CO_2^- \qquad\qquad\qquad (3.13)$$

$$CO_2^{\bullet-} + O_2 \rightarrow O_2^{\bullet-} + CO_2 \qquad\qquad\qquad (3.14)$$

3.5.2 The effect of carbon dioxide radicals on ascorbic acid oxidation

Solutions of 20 μM ascorbic acid and 0.2 M sodium formate in pH 7.0 phosphate buffer solution was pre-gassed with argon for 2 hours in Oxford pipettor before irradiation. Then the solution was separated in two parts: non-irradiated and irradiated. Samples were collected every 30 seconds for a total of 120 seconds. After irradiation, 26 units of catalase per ml were added into solution and incubated for 10 min at room temperature. The ascorbic acid absorbance was read directly at 255 nm by a spectrophotometer.

In the presence of argon, only hydroxyl radicals are produced, but not $O_2^{\bullet-}$. Under argon, only carbon dioxide radicals would be generated from irradiated sodium formate. The results in Figure 3.16 show that the amounts of ascorbic acid were maintained constant, with no ascorbic acid oxidized in the irradiated or control group. Since $O_2^{\bullet-}$ radicals, which can oxidize ascorbic acid, were not produced, the results show that $CO_2^{\bullet-}$ radicals did not induce ascorbic acid oxidation.
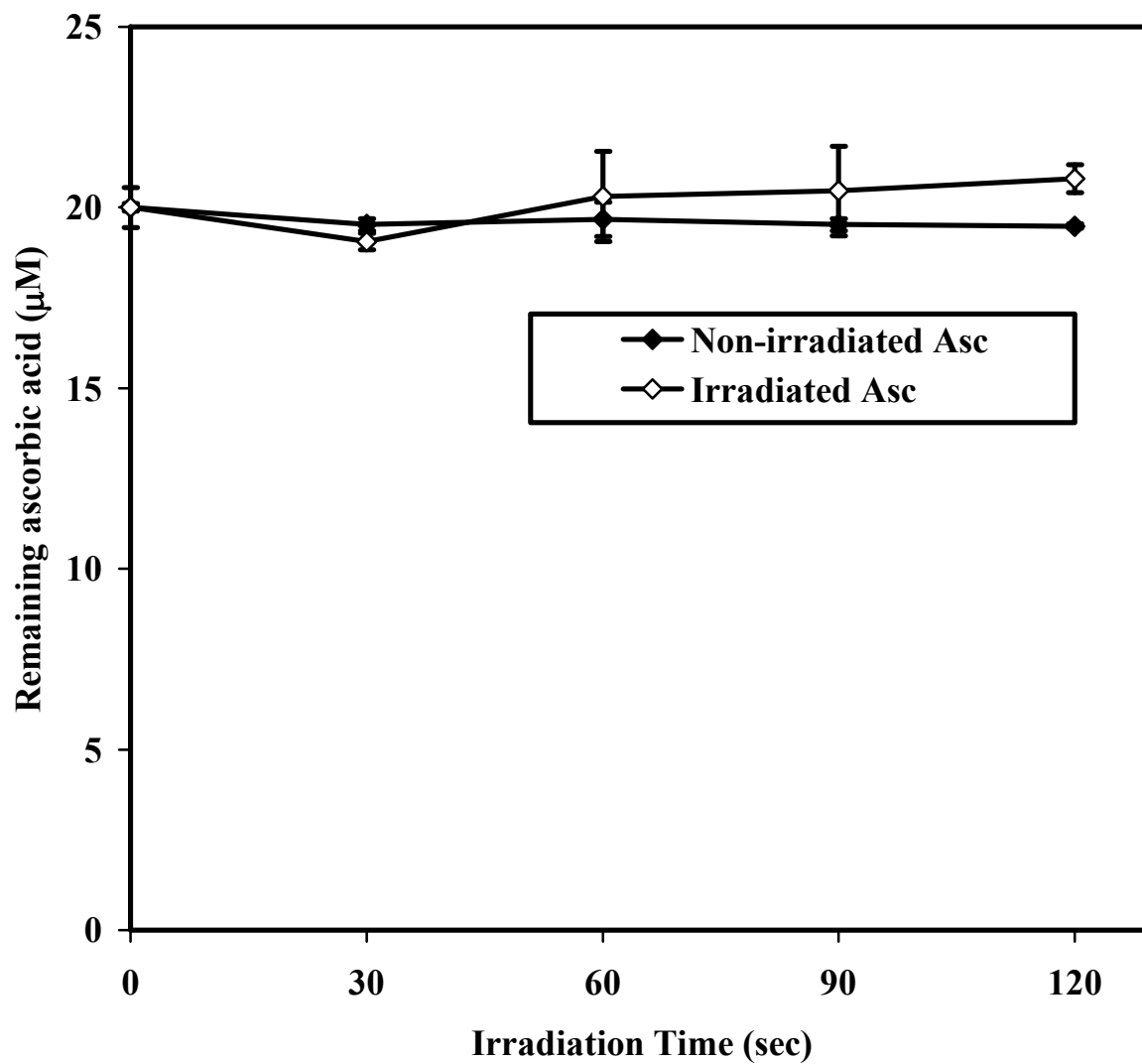
Figure 3.16 The effect of $CO_2^{\bullet-}$ radicals on ascorbic acid in the presence of argon

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method. Experimental details are in Section 3.5.2. The data represent the means of three replicates, with standard deviations as indicated by bars.

3.5.3 Effect of BSA on superoxide radical induced ascorbic acid oxidation

20 μM ascorbic acid and 0.2 M sodium formate with or without 500 μM BSA solutions were irradiated by gamma radiation for 0-120 seconds in 20 mM pH7.0 phosphate buffer. After irradiation, 26 units of catalase were added and the samples incubated for 10 min at room temperature. The protein was precipitated with 2 M perchloric acid on ice for 20 min and then centrifuged at 16,500 rpm for 20 min at 4 $^{\circ}$C. The supernatant of each sample was analyzed for ascorbic acid using an absorbance maximum of λ_{max} at 244.5 nm.

The rate constants of reaction of HO$^{\bullet}$ with sodium formate and BSA was 3.2×10^9 and 5×10^{10} dm^{3}m^{-1}s^{-1}, respectively (Buxton, Greenstock et al. 1988; Carr and Frei 1999). For 200 mM sodium formate and 0.5 mM BSA, the reaction rate ratio is 104. Therefore, 99% of HO$^{\bullet}$ generated with sodium formate. The level of oxidized ascorbic acid was 1.5 fold lower in formate only solution than in BSA-containing formate solution (Table 3.13).

Table 3.13 Effect of BSA on superoxide radical induced ascorbic acid oxidation after 120 second irradiation (39.7 Gy/min)

Substrate	BSA	Sodium formate	Oxidized ascorbic acid (μM)	Primary radicals	Secondary radicals
Asc	-	+	11.9	$O_2^{\bullet-}$	-
Asc	+	+	18.7	HO$^{\bullet}$	BSA$^{\bullet}$, BSAOO$^{\bullet}$

The results in Figure 3.17 indicated that both superoxide anion and BSA radicals can gradually induce ascorbic acid oxidation along with increasing irradiation time. The BSA$^{\bullet}$ and BSAOO$^{\bullet}$ radicals were less efficient oxidants of ascorbic acid than the $O_2^{\bullet-}$ under my experimental conditions.
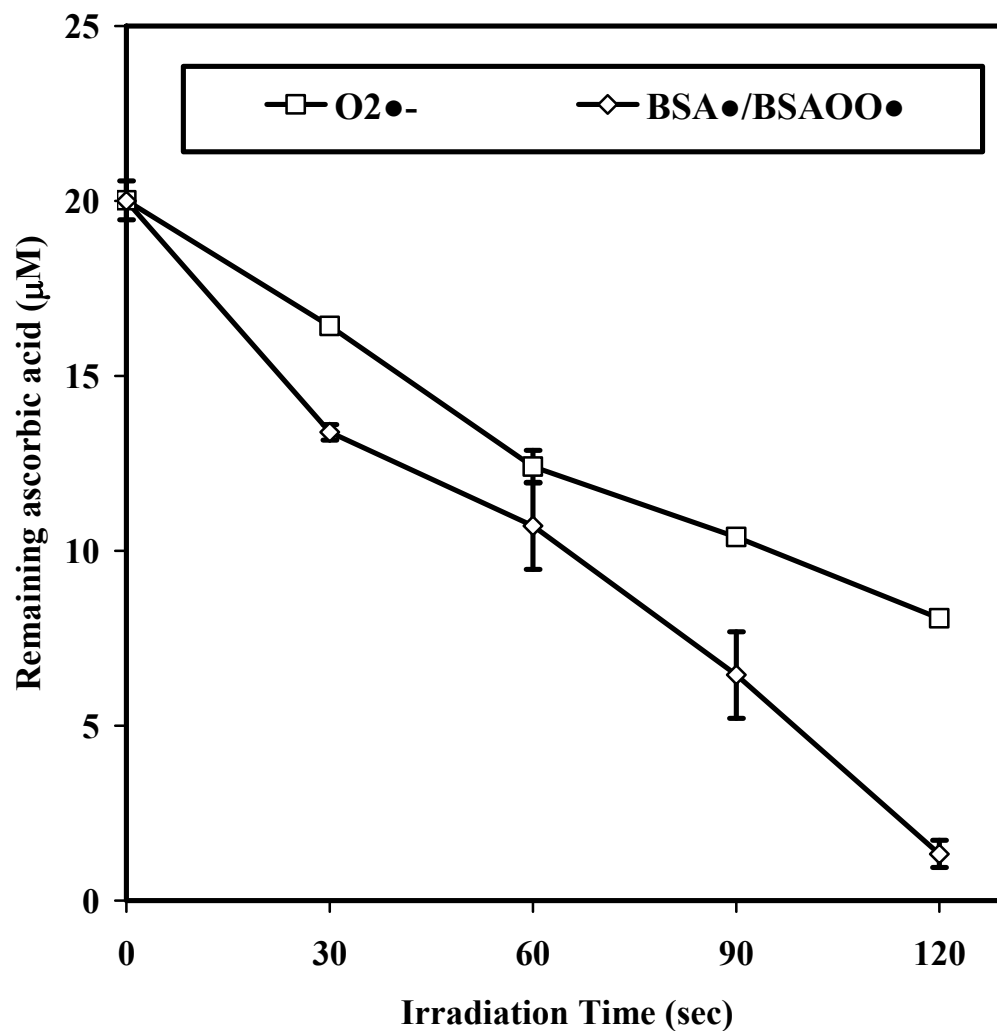
Figure 3.17 Effect of BSA on superoxide radical-induced ascorbic acid oxidation

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method. Experimental details are in Section 3.5.3. The data represent the means of three replicates, with standard deviations as indicated by bars.

3.6 Comparison of the actions of different radicals on ascorbic acid

3.6.1 Different radical generation

Hydroxyl radicals and superoxide anion ($HO^\bullet$ and $O_2^{\bullet-}$)

Irradiation of dilute solutions in water produces the oxidizing hydroxyl radicals.

$$H_2O \rightarrow e_{aq}^- + HO^\bullet + H^\bullet \qquad\qquad (3.15)$$

In the presence of O_2, superoxide and hydroperoxyl radicals are generated.

$$e_{aq}^- + O_2 \rightarrow O_2^{\bullet-} \qquad\qquad (3.16)$$

$$H^\bullet + O_2 \rightarrow HO_2^\bullet \qquad\qquad (3.17)$$

The pKa of the dissociation of $HO_2^\bullet$ is 4.8, so at pH 7 almost the secondary radicals are $O_2^{\bullet-}$.

Hydroxyl radicals ($HO^\bullet$)

In the presence of N_2O, the hydrated electrons are converted to hydroxyl radicals and the low $H^\bullet$ yield has no effect on the amount of products (Nauser 2005).

$$e_{aq}^- + N_2O + H_2O \rightarrow HO^\bullet + N_2 + OH^- \qquad\qquad (3.18)$$
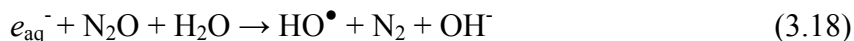
Because of their high reactivity, the $HO^\bullet$ radicals have been widely used to generate other radicals.

Protein radicals ($Pr^\bullet$)

In the case of protein, the effects of direct exposure to the $HO^\bullet$ have been extensively studied and include chain breakage, crosslinking and the formation of carbonyl group,

peroxides and hydroxylated amino acids. The protein peroxides are generated in reactions between protein radicals and dioxygen: here $X^\bullet$ is one–electron oxidant such as a hydroxyl ($HO^\bullet$) radical and $Pr^\bullet$ and $PrOO^\bullet$ are the protein carbon- and oxygen-centred radicals (Nauser, Koppenol et al. 2005):

$$PrH + X^\bullet \rightarrow Pr^\bullet + XH \qquad (3.19)$$

$$Pr^\bullet + O_2 \rightarrow PrOO^\bullet \qquad (3.20)$$

$$PrOO^\bullet + (H^+ + e^-) \rightarrow PrOOH \qquad (3.21)$$
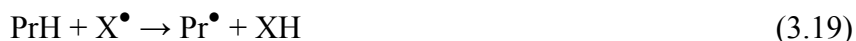
Azide radical ($N_3{}^\bullet$)

The azide reacts with $HO^\bullet$ generated by gamma irradiation to give an azide radical, $N_3{}^\bullet$, with rate constant of $1.2 \times 10^{10}\,M^{-1}s^{-1}$ (Davies 1997; Halliwell 1999).

$$N_3^- + HO^\bullet \rightarrow N_3{}^\bullet + OH^- \qquad (3.22)$$

Azide radicals are also strong oxidants, but with a much greater specificity than the $HO^\bullet$.

LZTrp$^\bullet$ / LZTyrO$^\bullet$ radicals

Radiation-generated azide radicals (reaction 3.22) can oxidize the side chain of tryptophan (Trp) to the indolyl radical ($Trp^\bullet$) in lysozyme with rate constant of $2 \times 10^9\,M^{-1}s^{-1}$ (Nauser, Koppenol et al. 2005).

$$N_3{}^\bullet + LZTrpH \rightarrow N_3^- + LZTrp^\bullet + H^+ \qquad (3.23)$$
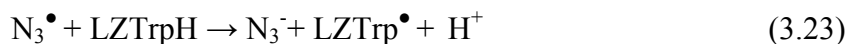
The azide radicals also rapidly oxidize the tyrosine side chain to the phenoxyl radical ($TyrO^\bullet$) with rate constant of $1.0 \times 10^8\,M^{-1}s^{-1}$. These reactions were first detected in hen egg white lysozyme (Stuart-Audette 2003). The radical products can be detected at different wavelengths, LZTrp$^\bullet$ at 510 nm and LZTyrO$^\bullet$ at 405 nm.

$$N_3^{\bullet} + LZTyrOH \rightarrow N_3^- + LZTyrO^{\bullet} + H^+ \qquad\qquad (3.24)$$

The rate constants for the reaction of different radicals with ascorbic acid and lysozyme are listed in Table 3.14. Using the rate constants, the concentration of azide and lysozyme in my experiments were set at 175 to 1000 fold higher than ascorbic acid (Table 3.15). Based on this design, the hydroxyl radicals reacted with azide (reaction 3.22) which produced secondary protein radicals. These secondary radicals could then oxidize ascorbic acid.

Table 3.14 Rate constants of reactions of selected radicals with ascorbic acid and lysozyme

Substrate	Radical type	Reaction rate ratio
Lysozyme / Asc$^-$	HO$^{\bullet}$	175
N$_3^-$/Asc$^-$	HO$^{\bullet}$	5.4×10^3
N$_3^-$/Lyspzyme	HO$^{\bullet}$	34

References: a: (Carr and Frei 1999);   b: (Halliwell 1999); c: (Stuart-Audette, Blouquit et al. 2003) ; and d: (Nauser 2005).

Table 3.15 Reaction rate ratio for reaction of selected radicals with ascorbic acid, azide and lysozyme

Substrate	Attacking radical	Rate constant (dm^{3}m^{-1}s^{-1})	Reference
Asc$^-$	HO$^{\bullet}$	1.1×10^{10}	a
Lysozyme	HO$^{\bullet}$	5.0×10^{10}	b
N$_3^-$	HO$^{\bullet}$	1.2×10^{10}	b
Asc$^-$	N$_3^{\bullet}$	4.0×10^9	b
Lysozyme	N$_3^{\bullet}$	9.6×10^8	c
LZTrpH	N$_3^{\bullet}$	2.0×10^9	d
LZTyrOH	N$_3^{\bullet}$	1.0×10^8	d

Concentration of substrates: 0.7 mM Lysozyme; 20 µM ascorbic acid and 0.1M azide.

3.6.2 Ascorbic acid measurement by the ferrozine assay

Ascorbic acid measurements at its peak absorbance in the UV can not be carried out in the presence of protein or azide. Effective removal of these compounds proved impossible so that alternative assay was needed to be developed.

3.6.2.1 The background of Ferrozine assay

Ferrozine is an effective chelator of ferrous iron and has been used for the determination of iron in biological samples. When it forms a complex with ferrous iron Fe^{2+}, it shows a characteristic UV-Vis absorption at 562 nm. Ascorbic acid can reduce ferric ion to ferrous which than reacts with ferrozine (light-yellow color) and produces the purple color with maximal absorbance in the visible range at 562 nm (Stookey 1970; Carter 1971).

3.6.2.2 Method of Ferrozine assay

Samples of 200 μl of test solutions and 225 μl water were mixed with 500 μl 0.1M sodium acetate-acetic acid buffer pH 4.0, 50μl 5 mM Fe^{3+} in the same buffer and 25μl 10mM ferrozine; after standing for 10 minutes at room temperature the purple color was fully developed. The absorbance at 562 nm was read in a spectrophotometer.

3.6.2.3 Ascorbic acid standard curve by ferrozine assay

Ascorbic acid solutions ranging from 0 to 50 μM were diluted from 500 μM ascorbic acid stock solution in water, and then 200 μl of each ascorbic acid solution was measured by the ferrozine assay. The standard curve was produced by plotting A_{562} against the

concentration of ascorbic acid (Fig. 3.18). The molar absorption coefficient calculated from this result was 1.12×10^4 l mol^{-1} cm^{-1}.

3.6.2.4 Ascorbic acid standard curve in 0.1M azide solution by ferrozine assay

The effect of azide on the ferrozine ascorbate assay was studied, because experiments were planned in which ascorbate was be oxidized in the presence of azide. Ascorbic acid solutions ranging from 0 to 50 µM were diluted from 500 µM ascorbic acid stock solution in 0.1 M azide, and then 200 µl of each ascorbic acid solution measured by the ferrozine assay. The standard curve was produced by plotting A_{562} against the concentration of ascorbic acid (Fig. 3.19). The molar absorption coefficient calculated from this result was 1.08×10^4 l mol^{-1} cm^{-1}.
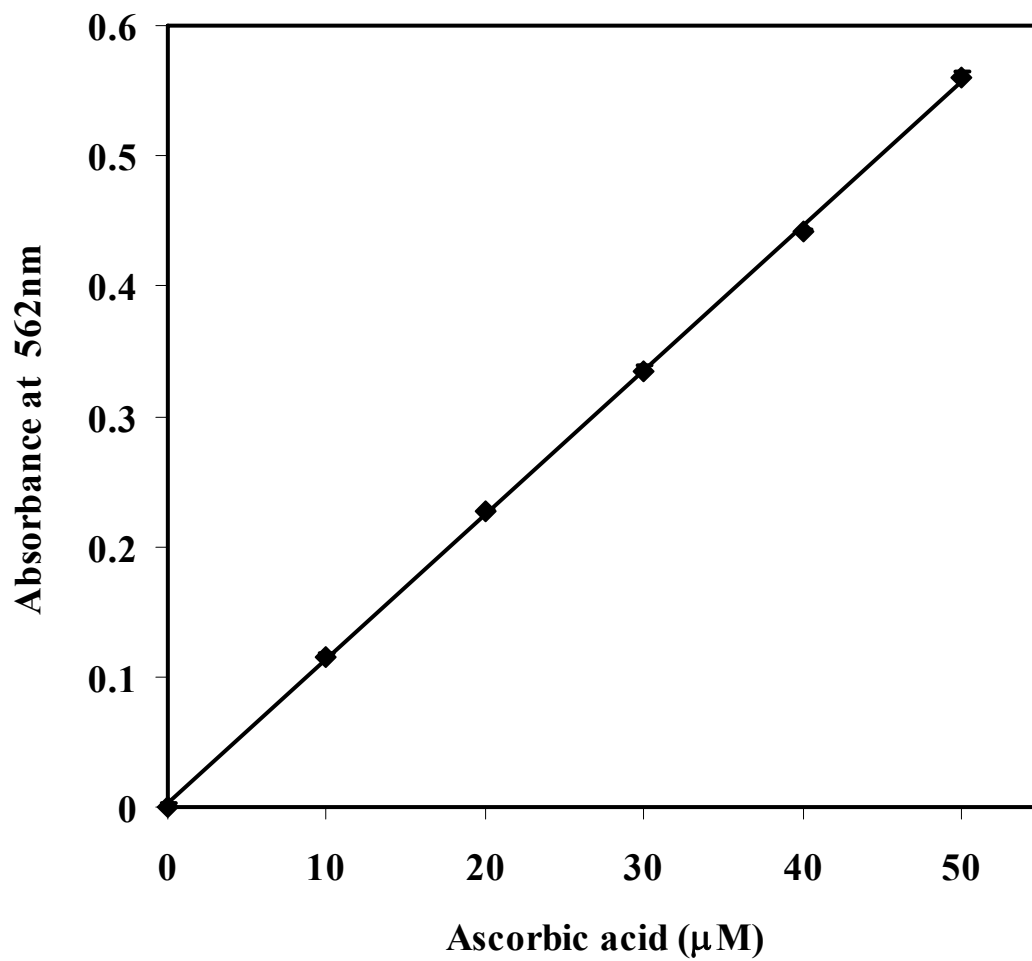
Figure 3.18 Standard curve of ascorbic acid in ferrozine assay

The ascorbic acid concentrations were measured by ferrozine assay. Experimental details are in Section 3.6.2.3. The data represent means ± SD of triplicates of a representative experiment. The SDs are smaller than the symbols representing the mean values. The correlation coefficient was 0.999.
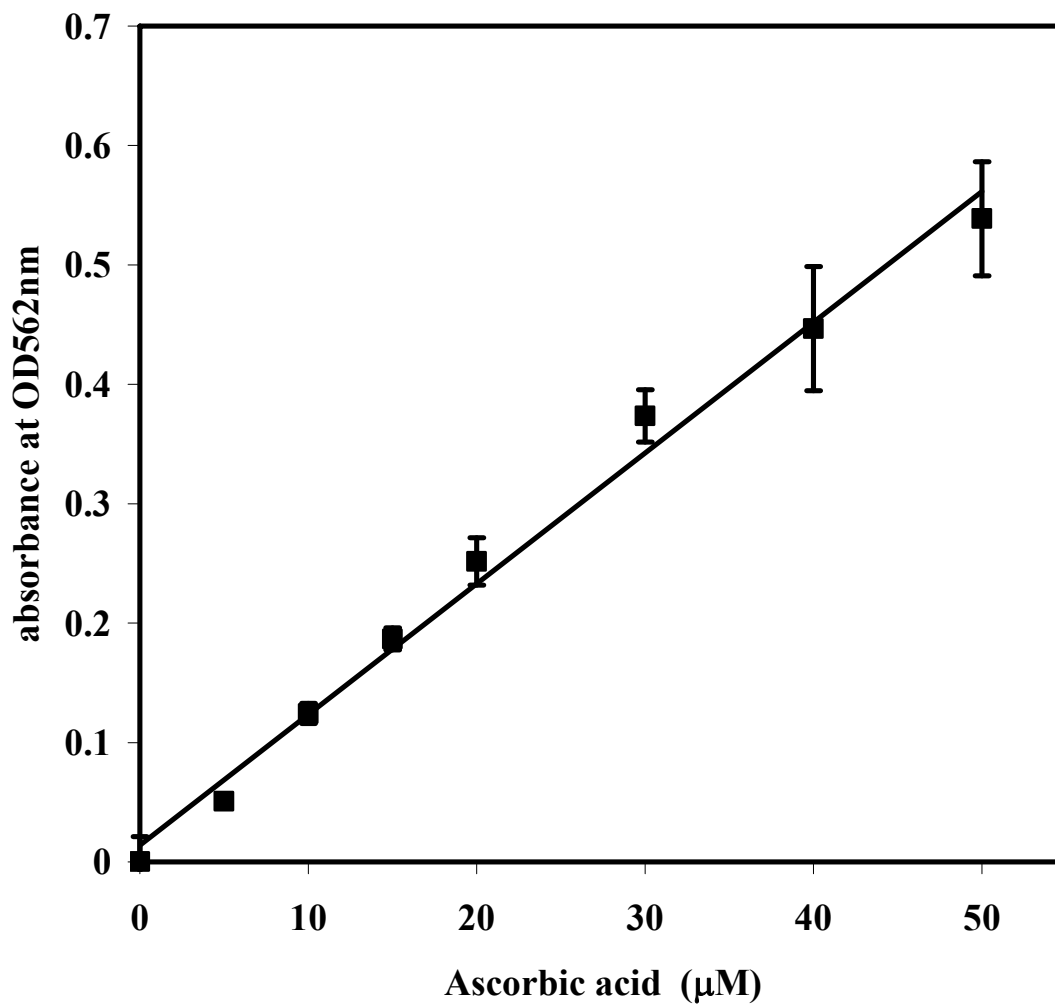
Figure 3.19 Standard curve of ascorbic acid with 100 mM azide in ferrozine assay

The ascorbic acid concentrations were measured by ferrozine assay. Experimental details are in Section 3.6.2.3.The data represent means ± SD of triplicates of a representative experiment. The correlation coefficient was 0.999.

3.6.3 Oxidation of ascorbic acid by different radicals

3.6.3.1 The effect of azide radicals on ascorbic acid oxidation

Different concentration of ascorbic acid solutions (0-50 µM) with 0.1M azide were irradiated by gamma radiation for 60 seconds with dose rate of 39.7 Gy/min. Ascorbic acid concentrations were measured by the ferrozine assay. In the presence of 0.1M azide, the radiation-generated $HO^\bullet$ radicals attack N_3^- to generate the azide radicals ($N_3^- + HO^\bullet \rightarrow N_3^\bullet$ + HO, $k = 1.2 \times 10^{10} M^{-1}s^{-1}$, Reaction 3.22). The results in Figure 3.20 show that the amount of ascorbic acid oxidation by azide radicals increased linearly in 0-20 µM ascorbic acid sample solutions. At higher ascorbic acid concentration (20-50 µM), the level of ascorbic acid oxidation appeared to plateau. Therefore, the amount of azide radical generated in this experiment oxidized 8.4 ± 0.8 µM ascorbic acid at the gamma dose of 39.7 Gy. The G value for $N_3^\bullet$ formation in the presence of dioxygen is 2.8 by pulse radiolysis (Land and Prutz 1979). Thus, both 11.5 µM $N_3^\bullet$ and 13.2 µM $O_2^{\bullet-}$ could only oxidize 8.4 µM ascorbate, because the rest were consumed in competing radical-radical reaction. The ascorbic acid concentration of 25 µM was chosen for the following experiments, because at that level some primary radicals were available for reaction with other solutes.
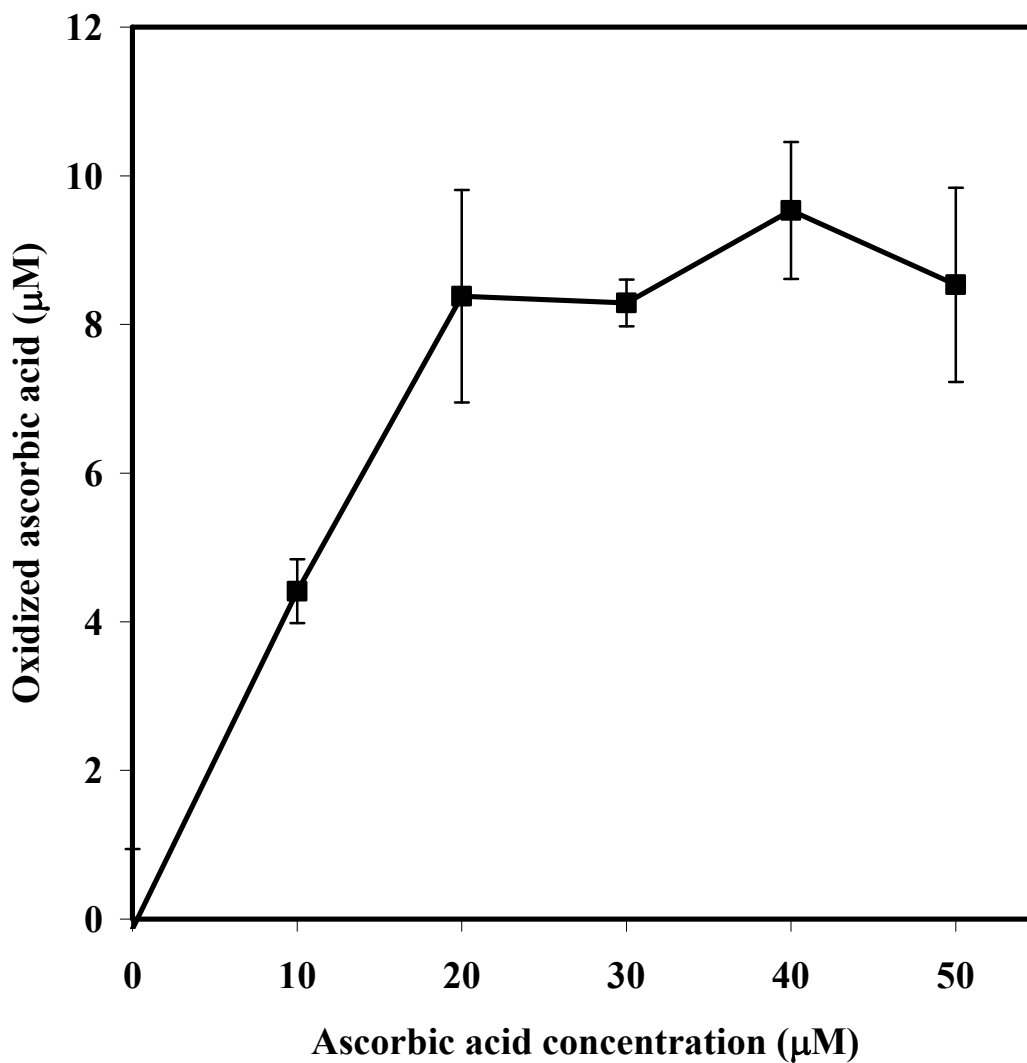
Figure 3.20   The effect of azide radicals on ascorbic acid oxidation

Ascorbate solutions in 0.1M sodium azide were irradiated with a dose of 39.7 Gy. The ascorbic acid concentration was measured by ferrozine assay as described in the Methods section. Experimental details are in Section 3.6.3.1. The data represent means ± SD of triplicates of a representative experiment.

3.6.3.2 Oxidation of ascorbic acid by azide and hydroxyl radicals

The 25 μM of ascorbic acid solution with or without 0.1 M azide was irradiated by gamma radiation for 90 sec under N_2O (59.6 Gy). The ascorbic acid concentration was measured by ferrozine assay. In the presence of 0.1 M azide, ascorbic acid was oxidized by azide radicals. The amount of oxidized ascorbic acid was significantly higher in samples exposed to hydroxyl radicals than to the azide radicals (Fig. 3.21). Superoxide radicals were also generated in similar amounts to $HO^{\bullet}$ and $N_3^{\bullet}$ (not shown).

3.6.3.3 Oxidation of ascorbic acid by lysozyme radicals

Different concentrations of ascorbic acid solutions in water with or without 0.7 mM lysozyme were irradiated by gamma irradiation for 90 sec (59.6 Gy). The results shown in Figure 3.22 indicate that the amount of oxidized ascorbic acid increased with increasing concentration of ascorbic acid in the absence or in the presence of lysozyme. However, the level of ascorbic acid oxidized in the presence of the protein was similar to that oxidized in its absence. This result showed that LZ protein radicals oxidized similar amount of ascorbic acid to hydroxyl radicals. In addition, the amount of oxidized ascorbic acid was maintained constant between 20 μM to 40 μM ascorbic acid in both groups. It showed that 20 μM ascorbic acid in solution was enough to scavenge protein and/or and hydroxyl radicals at a radiation dose of 59.6 Gy. The result also indicates that the presence of lysozyme had no effect on the ascorbate decay by ferrozine.
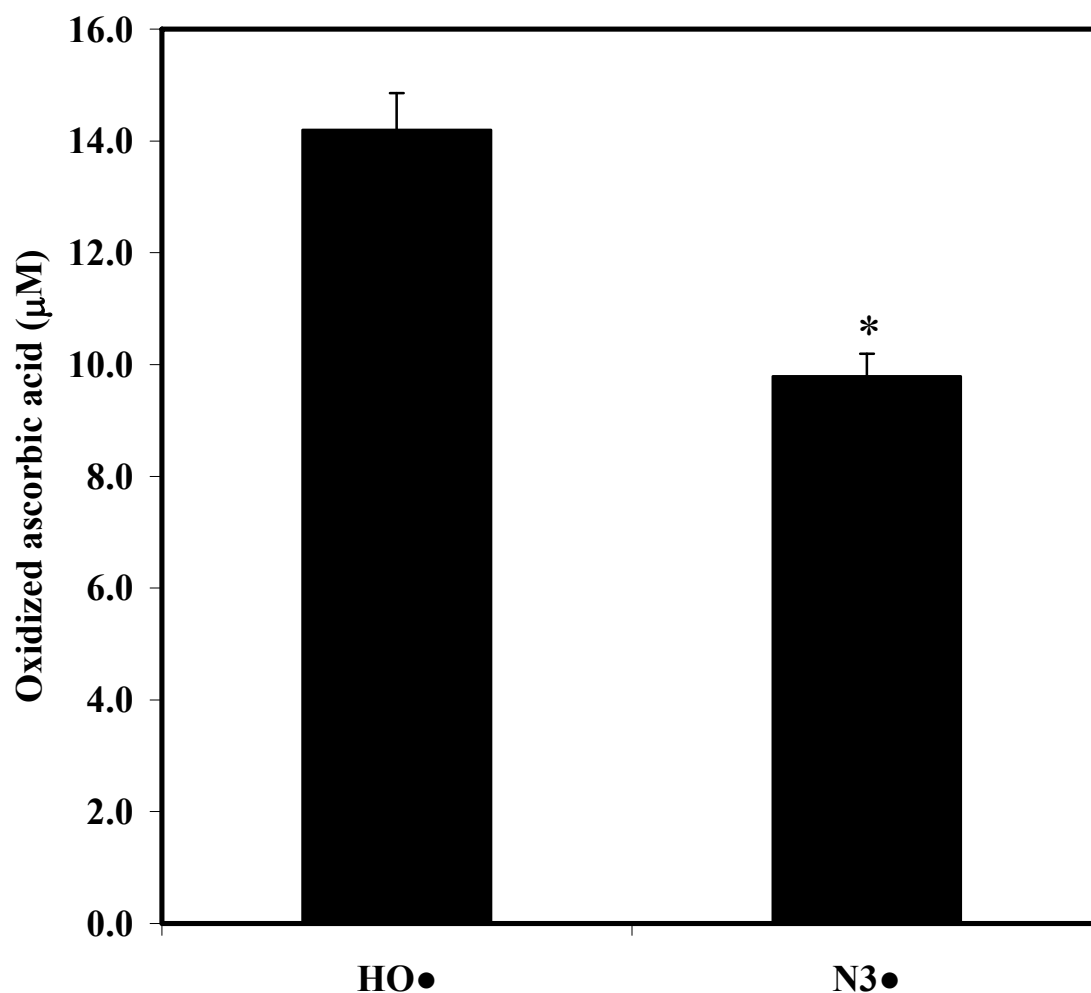
Figure 3.21 The amount of ascorbic acid oxidized by hydroxyl and azide radicals

The ascorbic acid concentration was measured by ferrozine assay as described in the Methods section and experimental details are in Section 3.6.3.2. The data represent means ± SD of triplicates of a representative experiment. *: indicates statistically significant difference between hydroxyl radical and azide radical samples at $p<0.05$ level. The bars include contribution from $O_2^{\bullet-}$ which may be as high as ≈ 8.0 μM of ascorbic acid oxidized.
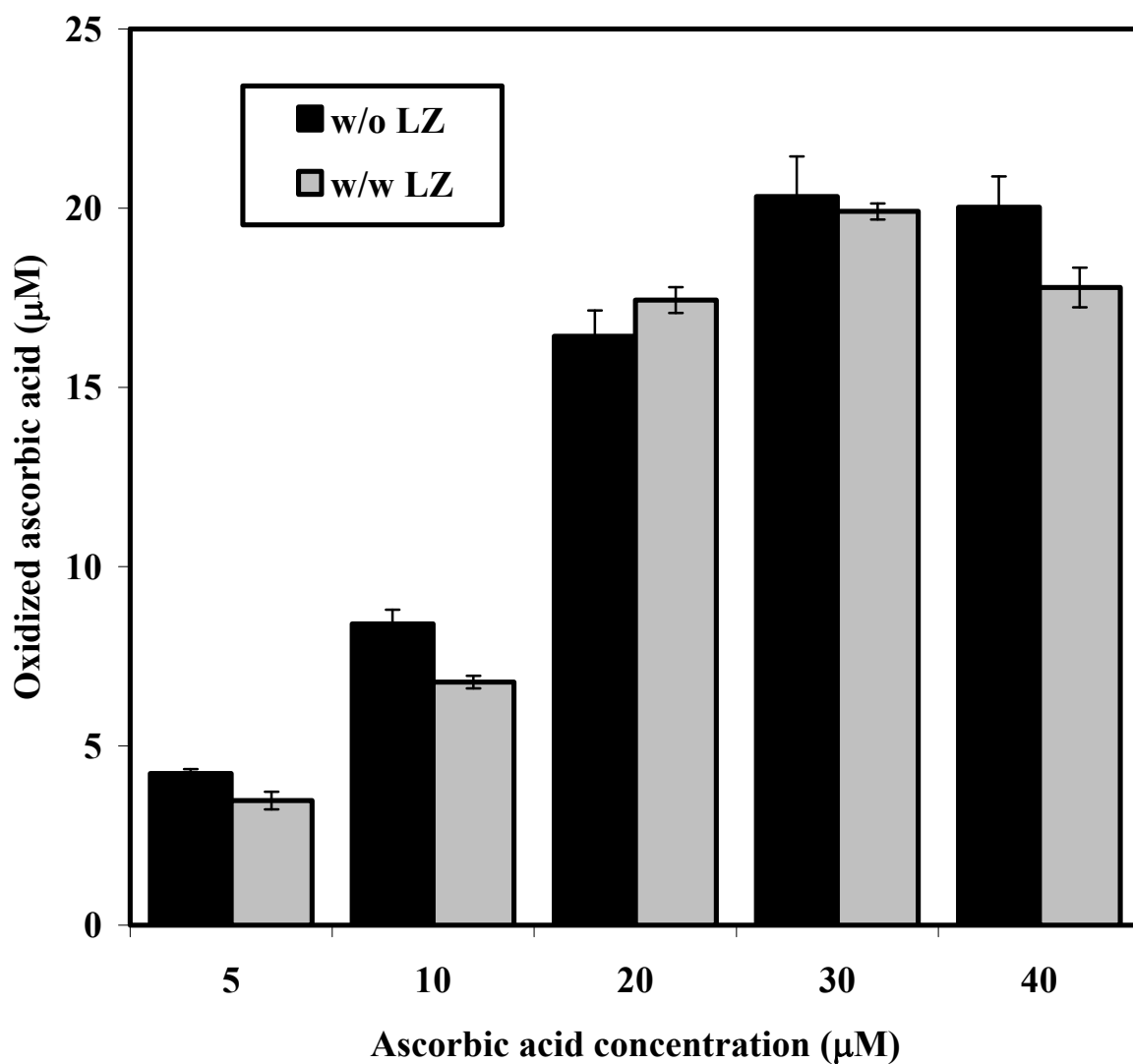
Figure 3.22 The effect of lysozyme in ascorbic acid oxidation

The ascorbic acid concentration was measured by ferrozine assay as described in the Methods section and experimental details are in Section 3.6.3.3. The data represent means ± SD of triplicates of a representative experiment. w/w: with; w/o: without; and LZ: lysozyme.

3.6.3.4 Comparison of different radicals as oxidants of ascorbic acid

Sample solutions containing 20 μM ascorbic acid, 0.1 M azide and 0.7 mM lysozyme were irradiated for 0, 30, 60, 90 and 120 sec with a gamma dose rate of 39.7 Gy/min. The ascorbic acid concentrations were measured by the ferrozine assay.

During irradiation, the initial oxidizing radicals were hydroxyl radicals ($HO^\bullet$) in all cases. These radicals were converted to the azide radicals ($N_3{}^\bullet$) when the solution contained scavenging concentration of azide; these generated lysozyme C- and O-centred protein radicals ($LZ^\bullet$/ $LZOO^\bullet$) when the solution consisted of lysozyme and $LZTrp^\bullet$/ $LZTyrO^\bullet$ when the solution contained both lysozyme and azide. The remaining amount of ascorbic acid was reduced with increasing radiation time in all four groups (Fig. 3.23). The kinetics of ascorbic acid oxidation were similar for all four radicals with only small differences. However, LZ protein radicals might be more effective than $HO^\bullet$ radicals. The reason for this is that Asc^- does not scavenge all $HO^\bullet$ radicals at the concentration used, but lysozyme does. The results also suggest that LZ protein radicals may be slightly more effective at ascorbic acid oxidation than $LZTrp^\bullet$/ $LZTyrO^\bullet$.
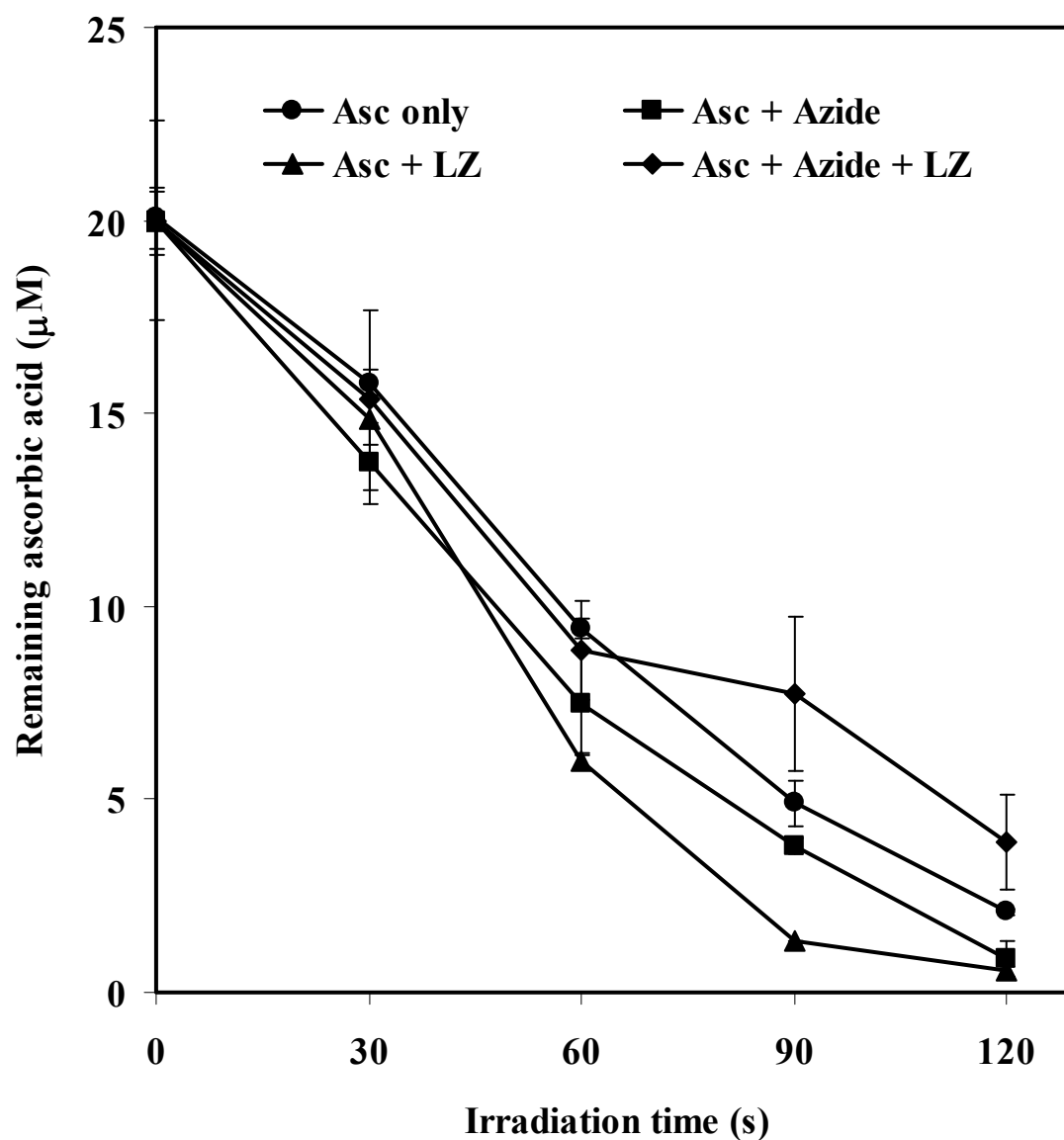
Figure 3.23 The effect of different radicals in ascorbic acid oxidation

The ascorbic acid concentration was measured by ferrozine assay as described in the Methods section and experimental details are in Section 3.6.3.4. The data represent means ± SD of triplicates of a representative experiment. Asc: 20μM; Azide: 0.1M; Lysozyme: 0.7mM. The results include contribution from $O_2^{\bullet-}$.

3.6.3.5 Comparison the effect of different radicals in ascorbic acid oxidation

Ascorbic acid with different reagents was irradiated for 60 sec with a gamma dose rate of 52 Gy/min in the presence of air or N_2O. Different reagents under different conditions produced different radicals: (1) $LZ^\bullet$ / $LZOO^\bullet$: 10μM Asc + 2mM lysozyme under air; (2) $LZ^\bullet$: 10μM Asc + 2mM lysozyme under N_2O, (3) $LZTrpOO^\bullet$: 10μM Asc + 2mM lysozyme + 5mM azide under air; (4) $LZTrp^\bullet$ /$LZTyrO^\bullet$: 10μM Asc + 2mM lysozyme + 5mM azide under N_2O. The ascorbic acid concentration was measured by ferrozine assay.

Comparison of the amount of ascorbic acid oxidized by lysozyme radicals generated by $HO^\bullet$ or $N_3^\bullet$ under N_2O or air in 60 sec irradiation is shown in Figure 3.24. The $N_3^\bullet$ were generated by radiation in a gamma source of 0.1 M azide solution saturated with N_2O, as in the fast kinetic experiments. The $HO^\bullet$ were produced similarly, but without the azide. In the absence of azide and dioxygen, the radiation-generated $HO^\bullet$ attacked the lysozyme to produce carbon-centred protein radicals, $LZ^\bullet$. Using azide and N_2O, all $HO^\bullet$ were scavenged to produce $N_3^\bullet$, followed by formation of $LZTrp^\bullet$ and $LZTyrO^\bullet$. Dioxygen is expected to react with carbon-centred species such as $LZ^\bullet$, although the relevant rate constant have not been measured for protein radicals. Under air saturation, roughly equal amounts of $HO^\bullet$ and $O_2^{\bullet-}$ radicals were generated by the radiation. The former reacted with the lysozyme to produce $LZ^\bullet$ and $LZTrp^\bullet$ radicals, whose most likely fate was conversion to the corresponding peroxyl radicals: $LZOO^\bullet$, and $LZTrpOO^\bullet$ (Table 3.16).

Table 3.16 identifies the protein radicals principally responsible for the oxidation of ascorbate under the different experimental conditions. The results show that the presence of

dioxygen significantly enhanced the amount of ascorbate oxidized by the protein radicals, with $LZ^\bullet$ / $LZOO^\bullet$ radicals oxidizing the largest amount of ascorbic acid in these four groups. Under N_2O, lysozyme was only oxidized only by either $HO^\bullet$ or $N_3^\bullet$ radicals. There are six tryptophan residues in lysozyme, Trp^{62}, Trp^{63} and Trp^{123} are main target of $N_3^\bullet$ and are the main contributors to the radicals transfer to 2 of the 3 Try radicals in the protein (Santus, Patterson et al. 2000). Assuming that LZ radicals oxidized 100 % of ascorbic acid, 85% was oxidized by $LZTrp^\bullet$ /$LZTyrO^\bullet$ radicals. This result indicates that the aromatic rings of the protein amino acids are the preferred sites for free radical attack.

Table 3.16 Oxidation of ascorbic acid by radiation-generated protein radicals (The G-values give the number of AscH molecules oxidized per 100 eV of energy absorbed by the solution)

Atmosphere	Azide	Primary radicals	Protein radicals	Oxidized Asc (μM)	G value
Air	-	$HO^\bullet$	$LZ^\bullet$ / $LZOO^\bullet$	8.0 ± 0.1	1.9
N_2O	-	$HO^\bullet$	$LZ^\bullet$/$LZTrp^\bullet$	5.7 ± 0.2	1.1
Air	+	$N_3^\bullet$	$LZTrpOO^\bullet$ /$LZTyrOO^\bullet$	7.0 ± 0.6	1.7
N_2O	+	$N_3^\bullet$	$LZTrp^\bullet$/$LZTyrO^\bullet$	4.8 ± 0.1	0.9

(10μM Asc; 2mM lysozyme; and 5mM azide)
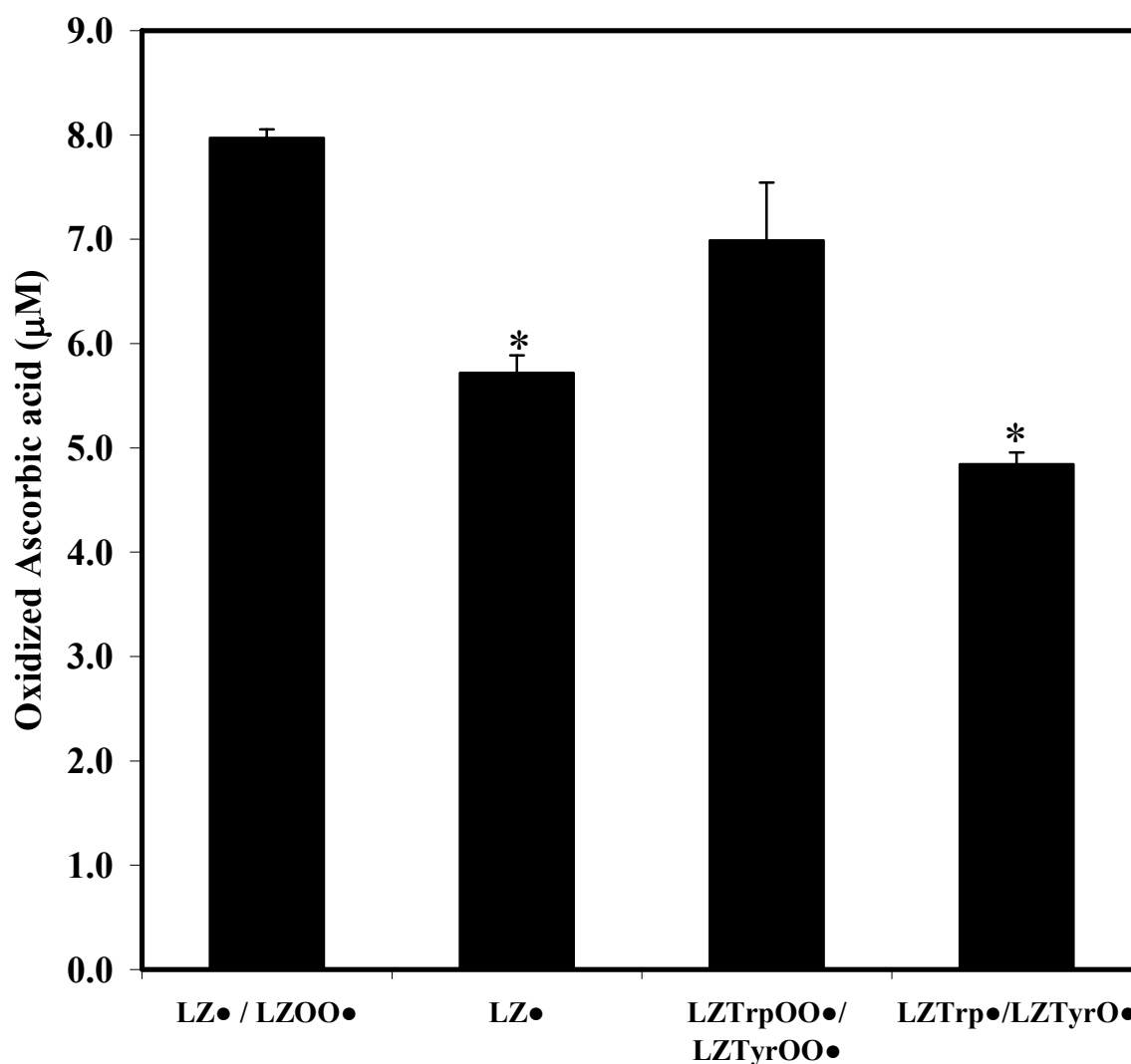
Figure 3.24 Comparison of different radicals in ascorbic acid oxidation by radiation dose of 52Gy

The ascorbic acid concentration was measured by ferrozine assay and experimental details are in Section 3.6.3.5.The data represent means ± SD of triplicates of a representative experiment. *: indicates statistically significant difference between LZ radicals and other radicals at $p<0.05$ level.

3.6.4 The effect of chymotrypsin protein radicals on ascorbic acid

Ascorbic acid (25 μM) with or without 25 mg/ml chymotrypsin was irradiated by gamma radiation for 0 and 90 seconds in the presence or absence of 0.1 M azide. The ascorbic acid concentration was measured by the ferrozine assay.

After 90 second irradiation, 64 % of the ascorbic acid was oxidized by $HO^{\bullet}$ radicals and 61.5 % by $Chy^{\bullet}/ChyOO^{\bullet}$ radicals (Table 3.17), but the difference was not significant. The $ChyTrpOO^{\bullet}$ radical was significantly less effective than the others (Fig. 3.25).

Table 3.17 The level of ascorbic acid oxidation in various sample solution after radiation in ferrozine assay

Sample solution	Radical	Oxidized Ascorbic acid	
		Concentration (μM)	Percentage of total (%)
25 μM Asc	$HO^{\bullet}$	16.1	64.3
25 μM Asc + 20 mg/ml chymotrypsin	$Chy^{\bullet}/ChyOO^{\bullet}$	15.4	61.5
25 μM Asc + 0.1 M azide	$N_3^{\bullet}$	10.5	42.1
25 μM Asc + 0.1 M azide + 20 mg/ml chymotrypsin	$ChyTrpOO^{\bullet}$	*6.1	*24.4

(*: $p<0.05$)

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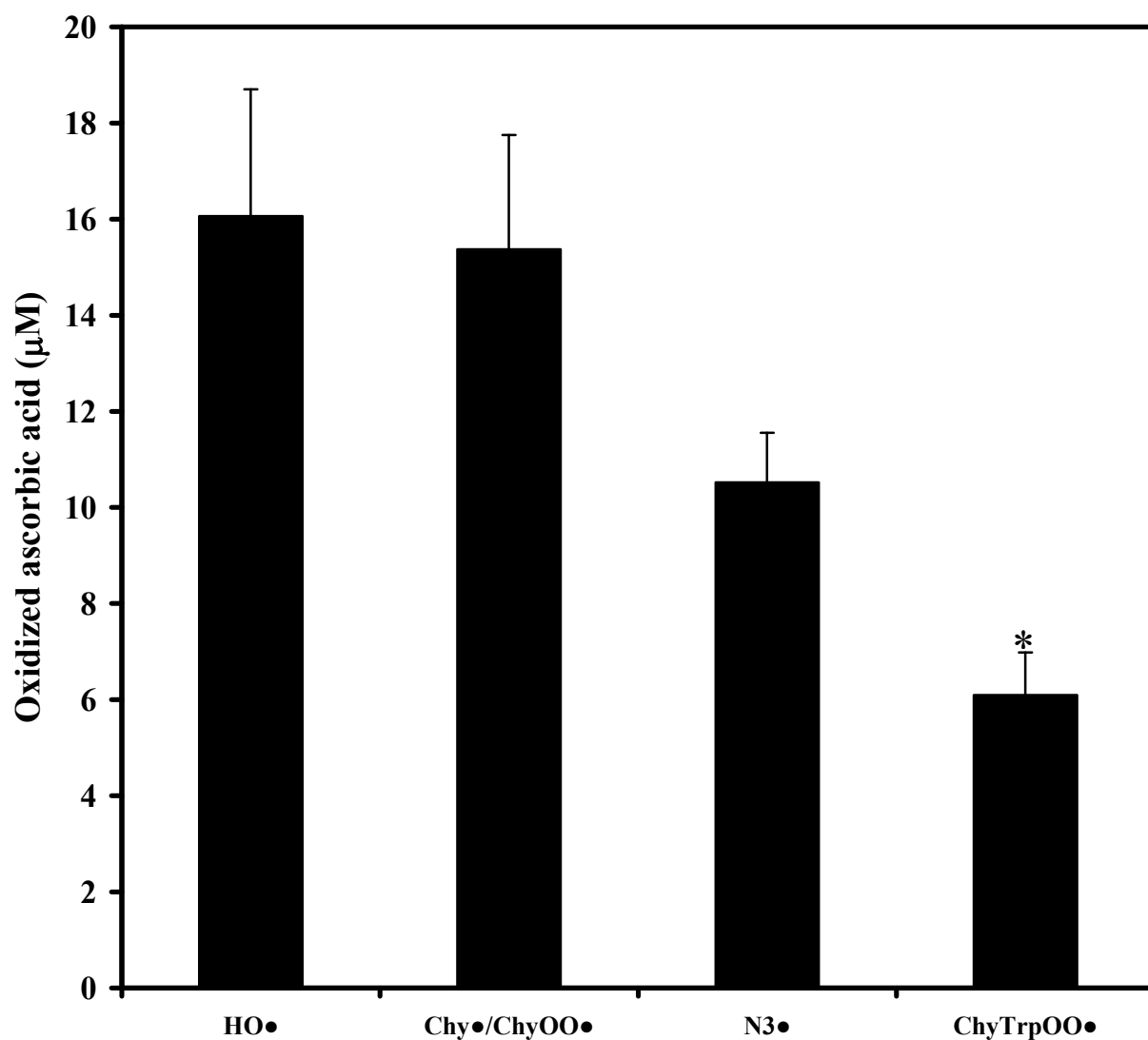
Figure 3.25 The comparison of hydroxyl, azide and chymotrypsin radicals on ascorbic acid oxidation

The ascorbic acid concentration was measured by ferrozine assay as described in the Methods section and experimental details are in Section 3.6.4. The data represent means ± SD of triplicates of a representative experiment. * Indicates statistically significant difference between hydroxyl radical and other radicals at $p<0.05$ level.

3.7 The effect of antioxidants on BSA radical-induced ascorbic acid oxidation and BSA peroxide formation

It has been postulated, that protein radicals are critical initial target of radicals in cells, able to propagate the damage to other molecules. The discovery of compounds able to repair the protein radicals before further damage would therefore provide substantial protections to the cell. In this section, a number of compounds were tested for their ability to protect ascorbate from oxidation by protein radicals.

3.7.1 The effect of antioxidants on BSA radical-induced ascorbic acid oxidation

TEMPO, TEMPOL, PBN, Rutin, Silibinin, and Trolox were used in these experiments. Various concentrations of the antioxidants with 20 μM ascorbic acid and 500 μM BSA were irradiated for 0 and 90 seconds in 20 mM phosphate buffer at pH 7.0. At the end of the radiation time, the sample was incubated with 26 units of catalase for 10 minutes at room temperature. The protein was precipitated with 2 M PCA on ice for 20 min and then centrifuged at 16,500 rpm for 20min at 4 ^{o}C. The supernatant of each sample was analyzed for ascorbic acid using an absorbance maximum of λ_{max} at 244.5 nm by a spectrophotometer.

In order to study the ability of various antioxidants in the protection of ascorbic acid from BSA radicals induced oxidation, we compared the amount of oxidized ascorbate generated in the presence of different antioxidants at the concentration of 100 μM. The results in

Figure 3.26 show that in both TEMPO and TEMPOL treatements, there was almost no oxidized ascorbate produced. This was surprising, because both these compounds are known to oxidize ascorbate directly. We assume that in our system this reaction was too slow to compete with that between the protein radicals and ascorbate. The addition of PBN, Rutin, and Trolox, the amounto of oxidized ascorbate was slightly redcued, but not signficant. The ascorbic acid oxidation was enhanced about 30 % by the addition of Silibinin. To conclude, only TEMPO and TEMPOL were able to significantly protect ascorbic acid from BSA radicals induced oxidation. PBN, Rutin, and Trolox have no effect in the inhibition of the ascorbate oxidation. Silibinin can induce acorbate oxidation.

3.7.2 The effect of antioxidants on BSA protein peroxide formation

3.7.2.1 The formation of BSA hydroperoxides by gamma irradiation

500μM BSA (1ml) was irradiated by gamma radiation at 0 to 5 minutes. At the end of the radiation time, the samples were incubated with 6 units/ml catalase for 10 minutes at room temperature. To each sample solution 55 μl of 0.5 M PCA, 25μl of 5mM XO and Fe^{2+} were added, followed by incubation in the dark for 40 to 60 minutes. Each sample was analyzed by the modified FOX assay using an absorbance maximum of λ_{max} at 560 nm by a spectrophotometer. The amount of protein hydroperoxide formation can be calculated by the equation (Gay, Collins et al. 1999):

$$\frac{\Delta 560}{35000} \times 10^6 \quad (\mu M)$$

Figure 3.27 was generated by plotting the amount of BSA hydroperoxide formation against the different irradiation times (gamma dose rate: 39.7 Gy/min).

According to Figure 3.27, the result showed that the amount of BSA hydroperoxide formation increased along with an increasing gamma dose rate. The curve is linear, with correlation coefficient of 0.993. The equation of the linear curve is:

$$[\Pr OOH] = \Delta Abs \times 2.877 - 0.274 \ (\mu M)$$

According to the equation, the amounts of BSA hydroperoxides at different radiation times can be calculated (Table 3.18). After 1 min radiation, 0.5 % of BSA peroxides were produced from 500 µM BSA in solution. The calculated maximum efficiency of the concentration of hydroxyl radicals to protein hydroperoxides in the presence of air was about 24 % (dose rate: 39.7 Gy/min).

Table 3.18 The formation of BSA hydroperoxides under air

Radiation Time (sec)	BSA hydroperoxide (µM)	Error! Bookmark not defined.Error! Bookmark not defined. $\left[\dfrac{BSAperoxide}{BSA}\right] \times 100$	$\left[\dfrac{BSAperoxide}{OHradicals}\right] \times 100$
0	0	0	0
30	1.2	0.2	21.0
60	2.6	0.5	23.4
90	4.0	0.8	24.3
120	5.5	1.1	24.7
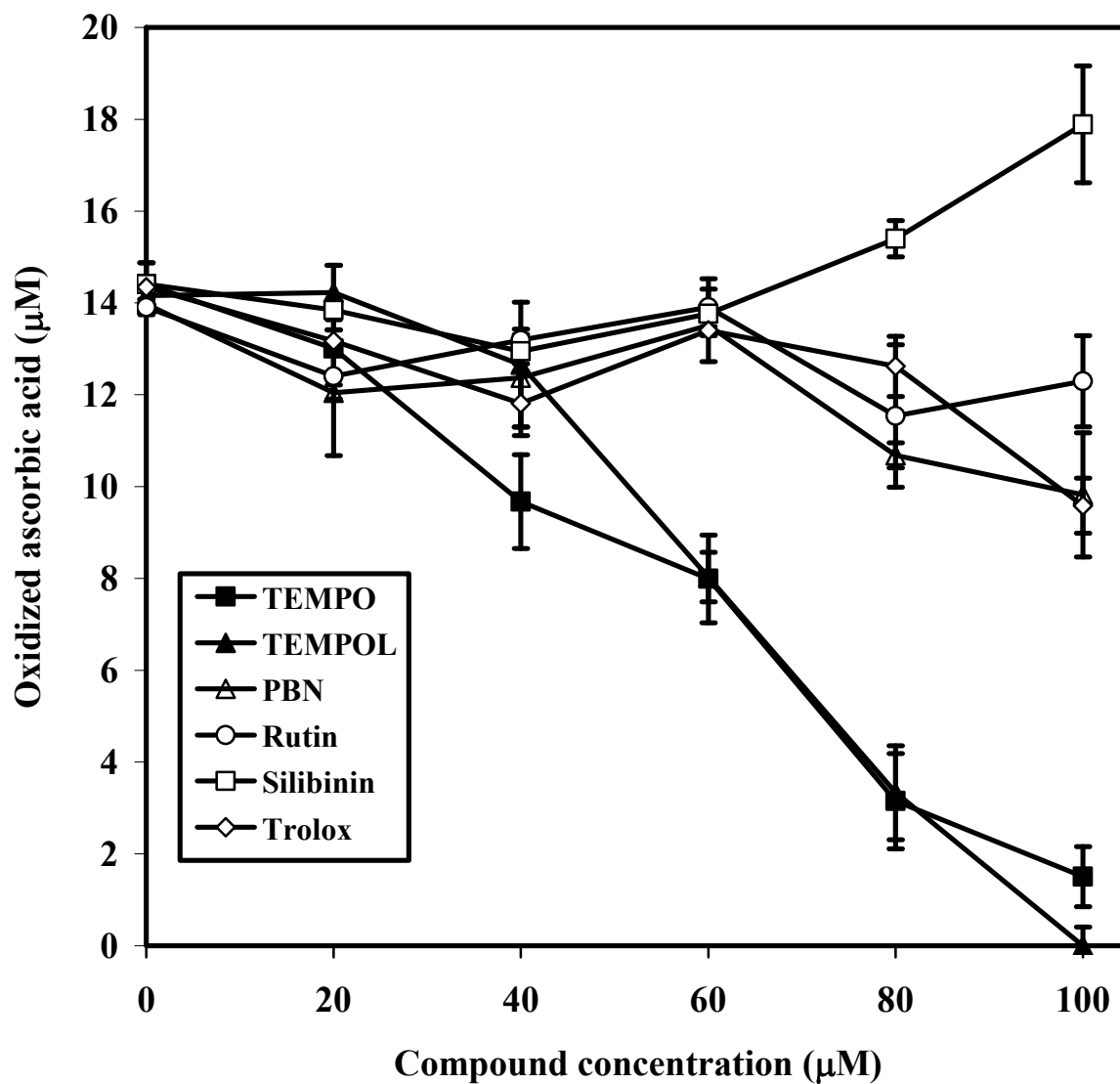
Figure 3.26 The effect of different antioxidants on BSA radical-induced ascorbic acid oxidation (59.6 Gy)

Ascorbic acid concentration was measured by the UV/Vis spectrophotometer method and experimental details are in Section 3.7.1.The data represent the means of three replicates, with standard deviations as indicated by bars.
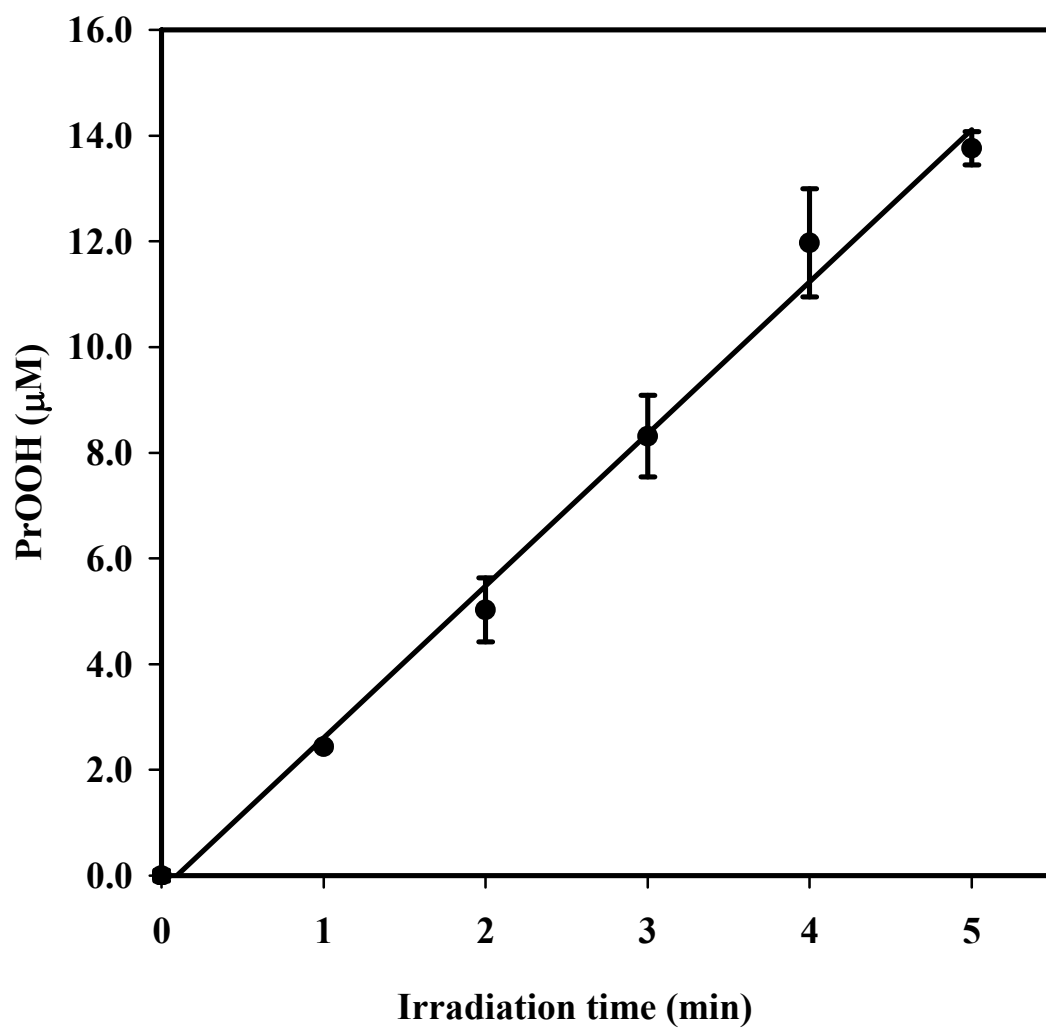
Figure 3.27 The formation of BSA hydroperoxides at different radiation time.

Protein hydroperoxide concentrations were measured by FOX assay and experimental details are in Section 3.7.2.1. The data represent the means of three replicates, with standard deviations as indicated by bars.

3.7.2.2 The effect of antioxidants on BSA hydroperoxide formation

Different concentrations of antioxidants: gallic acid, PBN, and trolox with 500μM BSA were irradiated by gamma radiation for 0 and 5 minutes. At the end of the radiation time, the sample was incubated with 6 units/ml catalase for 10 minutes at room temperature. To each sample solution 55 μl of 0.5 M PCA, 25μl of 5mM XO and Fe^{2+} was added, following by incubation in the dark for 40 to 60 minutes (modified FOX assay). Each sample was analyzed using an absorbance maximum of λ_{max} at 560 nm by a spectrophotometer.

The results shown in Figure 3.28 demonstrate that PBN had a minor protective effect on the BSA peroxide formation, while 60 μM Trolox reduced BSA peroxide formation by 15 %. Gallic acid was the most effective inhibitor of BSA peroxide formation.
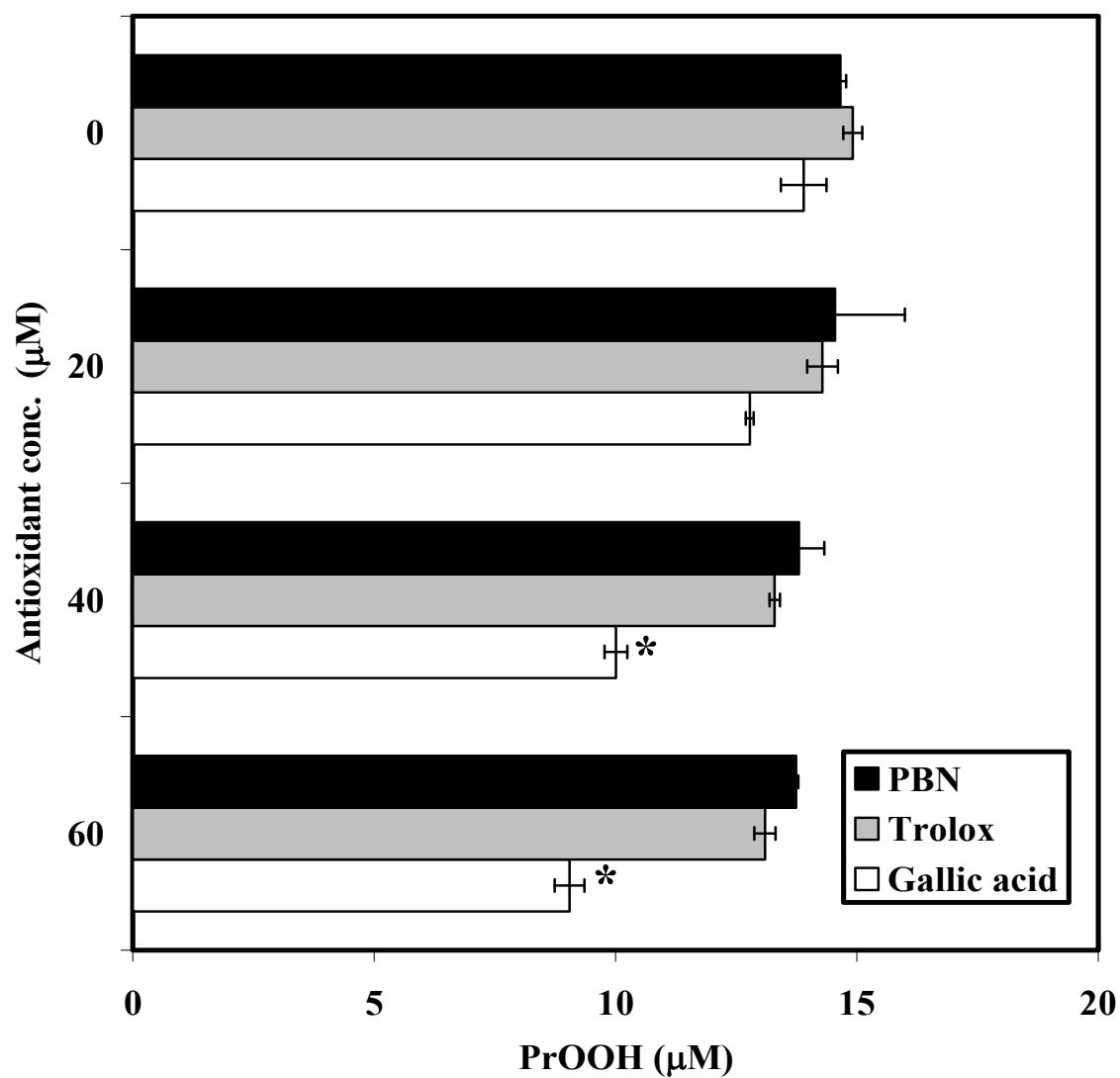
Figure 3.28 The effect of different antioxidants on BSA peroxide formation

Protein hydroperoxide concentrations were measured by FOX assay and experimental details are in Section 3.7.2.2. The data represent the means of three replicates, with standard deviations as indicated by bars. * Indicates statistically significant at *p*<0.05 level.

SECTION (II) CELL CULTURE STUDIES

3.8 Effect of gamma irradiation-induced oxidative damage to HL-60 myeloid leukaemia cells

3.8.1 The growth pattern of HL-60 cells

Growth is the process during which living microorganisms undergo cell division and consequently an increase in population size in mammalian cell culture. The growth of a population of cells grown in a closed environment typically shows four stages: lag phase; exponential phase; stationary phase; death phase. The lengths and characteristics of these phases will depend upon factors such as the nature of the growth medium and temperature of incubation. Growth rate constant, k, is a measure of the number of generations (the number of doublings) that occur per unit of time in an exponentially growing culture.

$$ k \; = \; \frac{\ln 2}{g} $$

where ln 2 is the natural log of 2 and g is the time in hours taken for the population to double during the exponential phase of growth (Foa, Maiolo et al. 1982).

Generation of a growth curve can be useful in evaluating the growth characteristics of a cell line. From a growth curve, the lag time, population doubling time and saturation density can be determined. The doubling time was calculated from serial cell count. The viable cell numbers were determined by trypan blue exclusion.

HL-60 cells were centrifuged at 3,000 rpm for 5 min at room temperature and the medium was carefully discarded. The pellet was resuspended in 5 ml of RPMI-1640 medium and the cells counted by hemocytometer. The cell suspension was diluted in order to have an appropriate amount of medium and cells to achieve a seeding density of 1×10^5 cell/ml. After mixing, the cells werer transferred to a 25-cm^2 flask. The leftover cell suspension was counted in order to determine the actual seeding density. The flask was placed at 37 ^{o}C in a humidified atmosphere of 5 % CO_2/ 95 % air. Cell numbers were counted every 24 hours and the results plotted on a log-linear scale. The population-doubling time can be determined by identifying a cell number along the exponential phase of the curve, tracing the curve until that number has doubled, and calculating the time. Cell number was plotted against incubation time on the graph (Fig. 3.29). Doubling time of the number of cells can be read from the graph. Proliferation in culture was exponential up to a saturation density of 1.4×10^6 cells/ml, with a doubling time of 33.6 hr. The growth rate constant, k, was then calculated as 0.021 hr^{-1}.
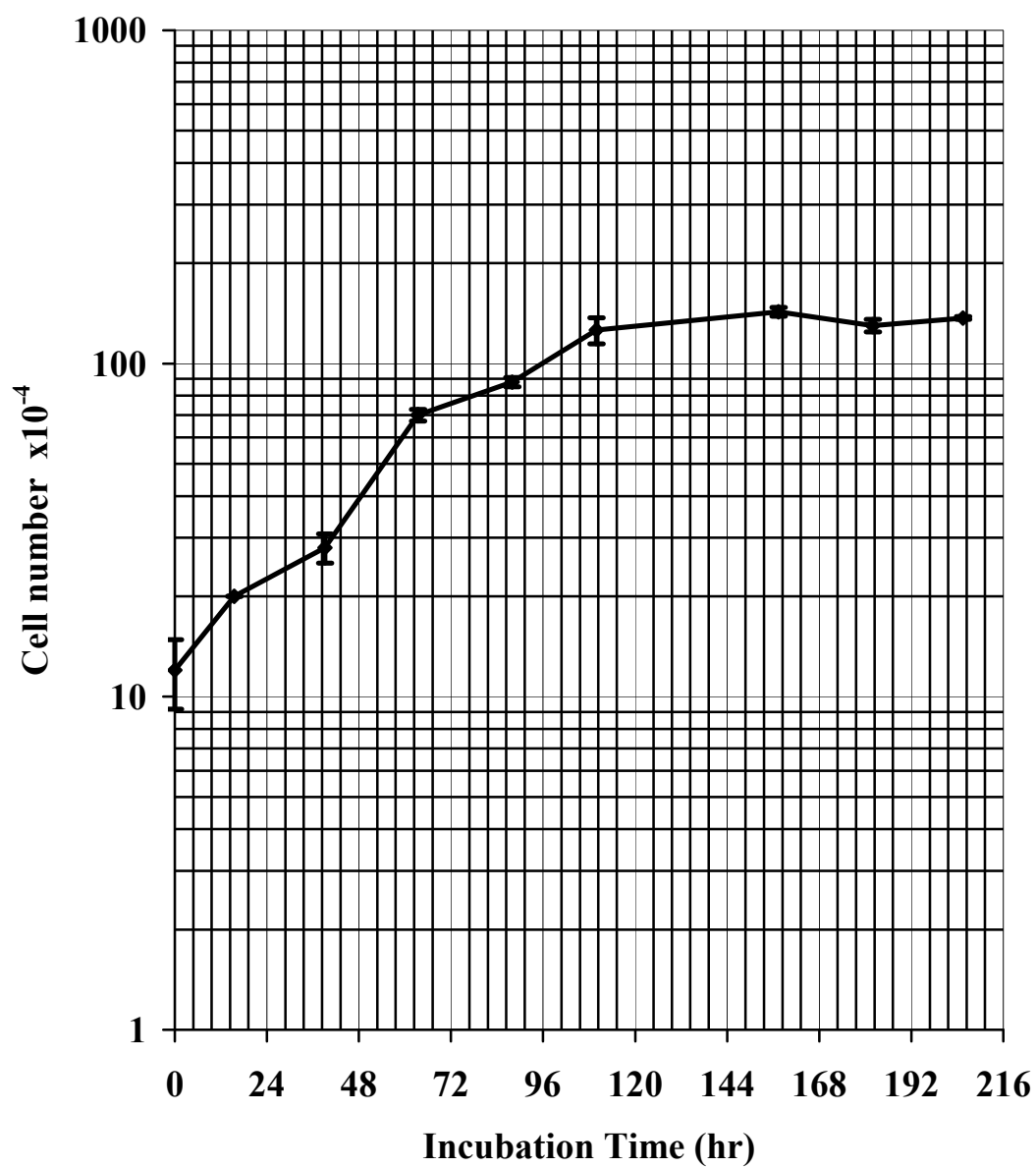
Figure 3.29 The growth curve of HL-60 cells in RPMI-1640 medium

Experimental details are in Section 3.8.1. The data represent the means of three replicates, with standard deviations as indicated by bars.

3.8.2 Effect of medium on γ-irradiation induced oxidative damage to HL-60 cells

3.8.2.1 Cell viability during irradiation in HEPES buffer and in RPMI-1640

HL-60 cells in exponential growth phase were harvested from serum-containing RPMI-1640 medium and suspended in the HEPES buffer or in serum-free RPMI-1640 medium. Then the cells were irradiated at room temperature for 0 and 20 min at dose rate of 45 Gy/min. The irradiation conditions were described in Methods. At both time points, the viability of cells was measured by both trypan blue exclusion assay and MTT assay.

A. Trypan Blue Exclusion assay

As shown in Figure 3.30, during 20 min irradiation in the HEPES buffer, the viability of the irradiation cells dropped significantly to 41.8 % ($p<0.005$), while control cells dropped to 89.6 %. The cell viability was maintained above 90 % in both control and irradiated cells in RPMI-1640 medium.

B. MTT assay

The MTT [3-(4,5)-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide] assay provides an indirect measurement of mammalian cells survival and proliferation, based on the ability of mitochondria to covert the pale yellow water-soluble tetrazolium MTT into a purple formazan product that is insoluble in aqueous solution. Live, growing cells have competent mitochondrial function that can actively reduce MTT to formazan. Conversely, dead or dying cells do not have this ability. The amount of MTT-formazan produced can be

determined spectrophotometrically once the MTT-formazan has been dissolved in a suitable solvent (Hansen, Nielsen et al. 1989).

The method of MTT assay to measure cell viability was described in Method section. The results in Figure 3.31, during 20 min irradiation in the HEPES buffer, show that the viability of the irradiated cells decreased significantly from 100 % to 38.9 % ($p <0.05$), while control cells maintained 100 %. The cell viability was slightly reduced to 78.5 % in irradiated cells in RPMI-1640 medium, but this was not statistically significant.

C. Comparison

Both cell viability assays showed that the statistically significant loss of viability in both control and irradiated cells was probably due to their suspension in HEPES buffer, not in RPMI-1640 medium (Table 3.19).

Table 3.19 Comparison of cell viability in different medium under different conditions

	Trypan Blue Exclusion		MTT assay	
	HEPES Buffer	**RPMI-1640**	**HEPES Buffer**	**RPMI-1640**
Control	89.6 ± 4.4 %	94.0 ± 2.2 %	100.0 ± 3.1 %	100.1 ± 0.1 %
Irradiated	* 42.8 ±15.2 %	90.7 ± 3.6 %	*38.9 ± 2.9 %	78.5 ± 5.8 %

*: $p<0.05$ indicates statistically significant difference between control and irradiated samples.
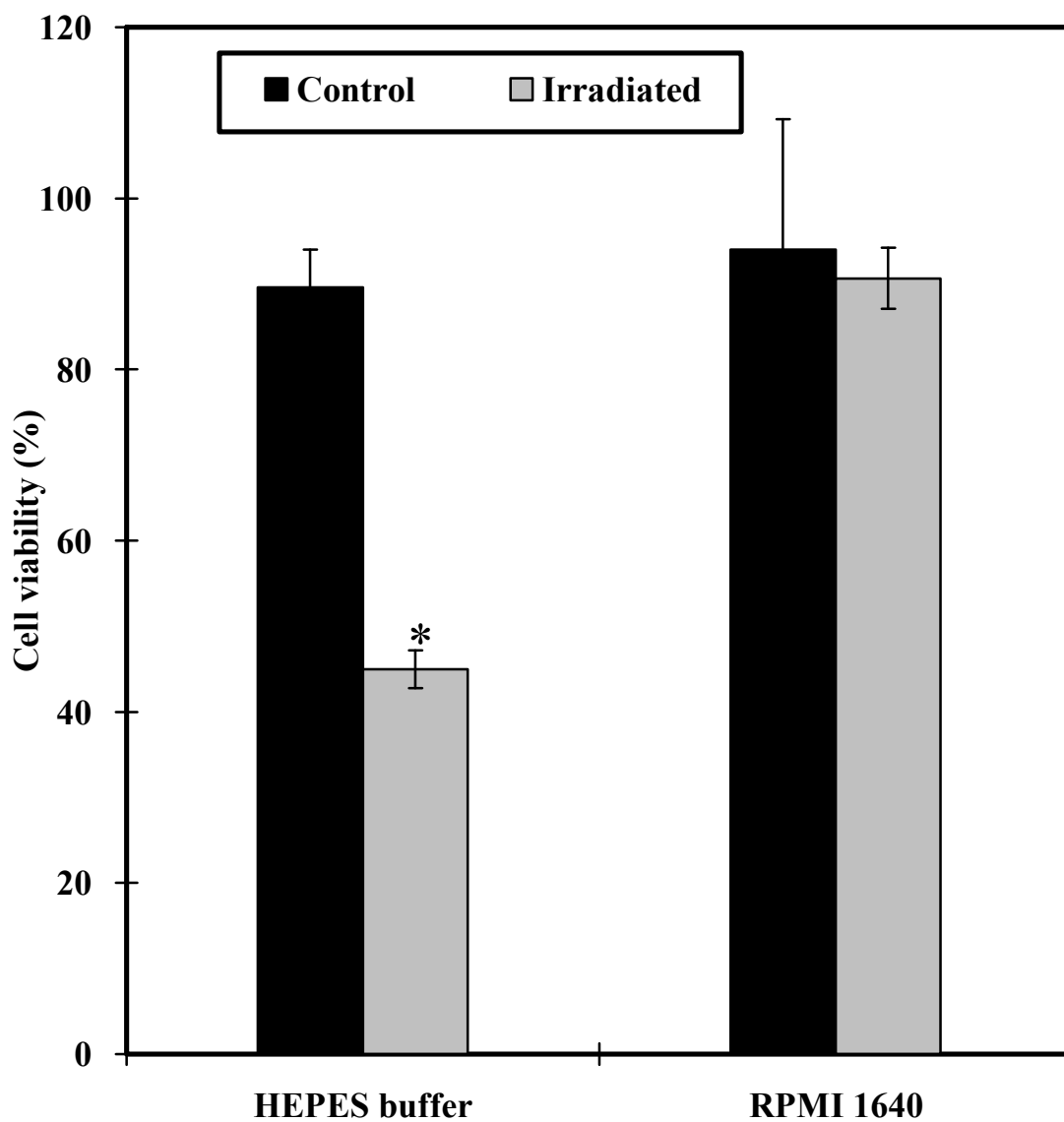
Figure 3.30 Effect of irradiation in HEPES buffer and in RPMI-1640 on cell viability using Trypan Blue Exclusion assay

The viability of cells was measured by trypan blue exclusion assay and experimental details are in Section 3.8.2.1. The data points were derived from five experiments. * indicates statistically significant difference between control and irradiated samples at $p<0.05$ level.
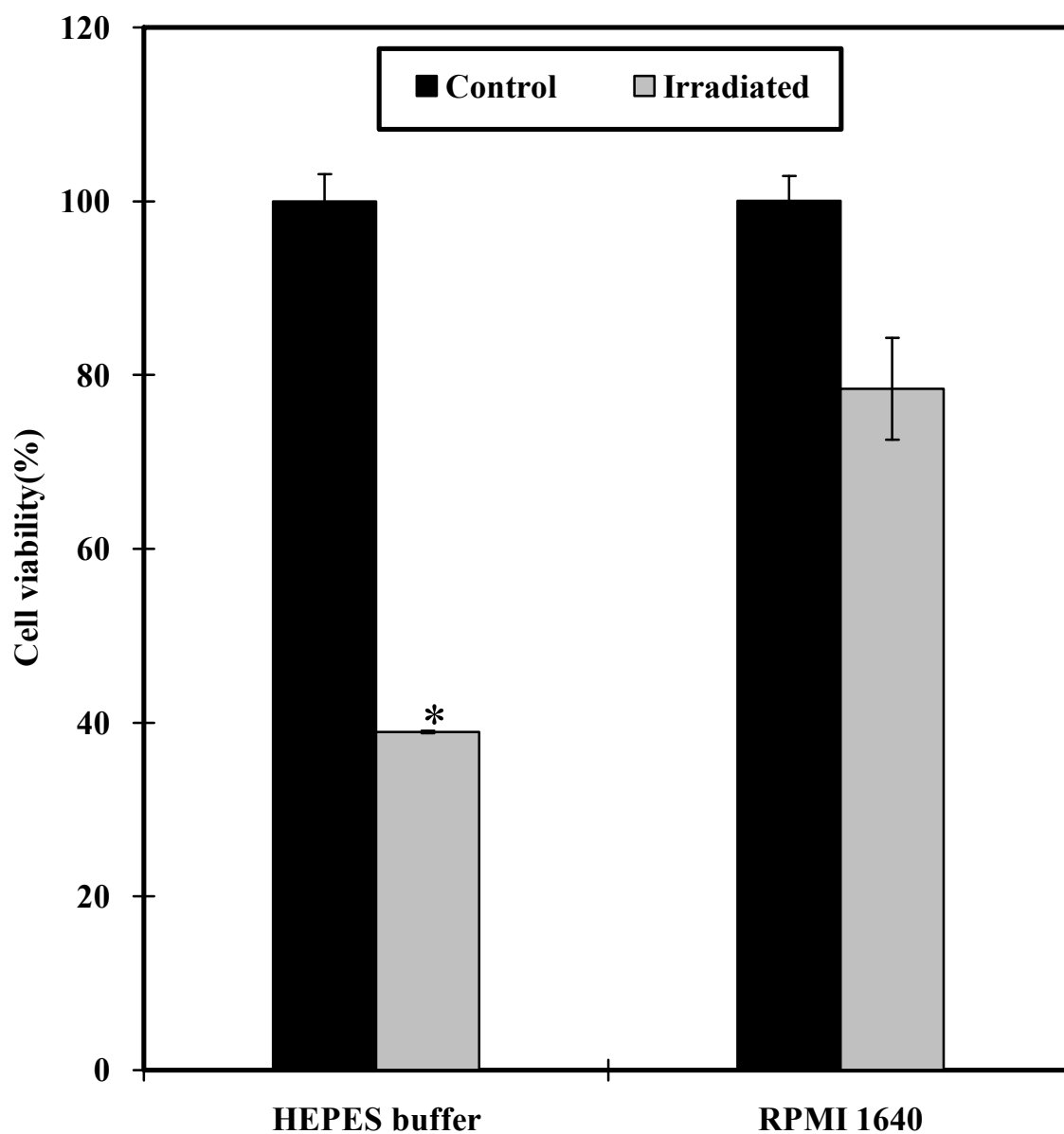
Figure 3.31 Effect of irradiation in HEPES buffer and in RPMI-1640 on cell viability using MTT assay

The viability of cells was measured by MTT assay and experimental details are in Section 3.8.2.1. The data points were derived from four experiments. * indicates statistically significant difference between control and irradiated samples at $p < 0.05$ level.

3.8.2.2 Protein peroxide formation in HL-60 cells irradiated in different media

3.8.2.2.1 The G-PCA-FOX assay

A range of hydroperoxides was reduced by ferrous ions in acid solutions and the amount of ferric product was measured as a xylenol orange complex at 560 nm. The hydroperoxides react with an excess of Fe^{2+} at low pH in the presence of the dye xylenol orange (XO) and the amount of Fe^{3+} generated is measured as the Fe–XO complex in the visible absorbance of 560 nm. Guanidine hydrochloride is used to keep the cell contents from precipitating when perchloric acid is added (Gay and Gebicki 2003).

3.8.2.2.2 G-PCA-FOX assay of intracellular hydroperoxides

HL-60 cells in exponential growth phase were harvested from serum-containing RPMI-1640 medium and suspended in the HEPES buffer or serum-free RPMI-1640 medium at a concentration of 2.5×10^6 cells/ml. Cells were irradiated in 60-mm diameter petri dishes (the depth of the medium kept within the range 2-5 mm) at room temperature for 0 and 20 min (dose rate of 45 Gy/min). After irradiation, the cells were washed with PBS before G-PCA-FOX assay as described in Method section. The concentrations of protein hydroperoxides were calculated with the molar absorption coefficient of 36,000 $M^{-1}cm^{-1}$.

The protein peroxide formation in the cells was significantly enhanced by irradiation in both medium systems ($p < 0.05$) (Fig. 3.32). The concentration of PrOOH produced can be calculated by the difference of absorbance before and after the irradiation, which is shown in Table 3.20. There was no significant difference in the amount of protein peroxides formed in cells irradiated in HEPES buffer and in RPMI-1640 media.

Table 3.20 Effect of irradiation medium in protein peroxide formation

HL-60 cells	ΔA_{560}	PrOOH (μM)
HEPES buffer	0.097 ± 0.004	2.771 ± 0.125
RPMI-1640 media	0.094 ± 0.025	2.695 ± 0.725

The cell number is 2.5×10^6 cells/ml in each original solution.

PrOOH: protein peroxides
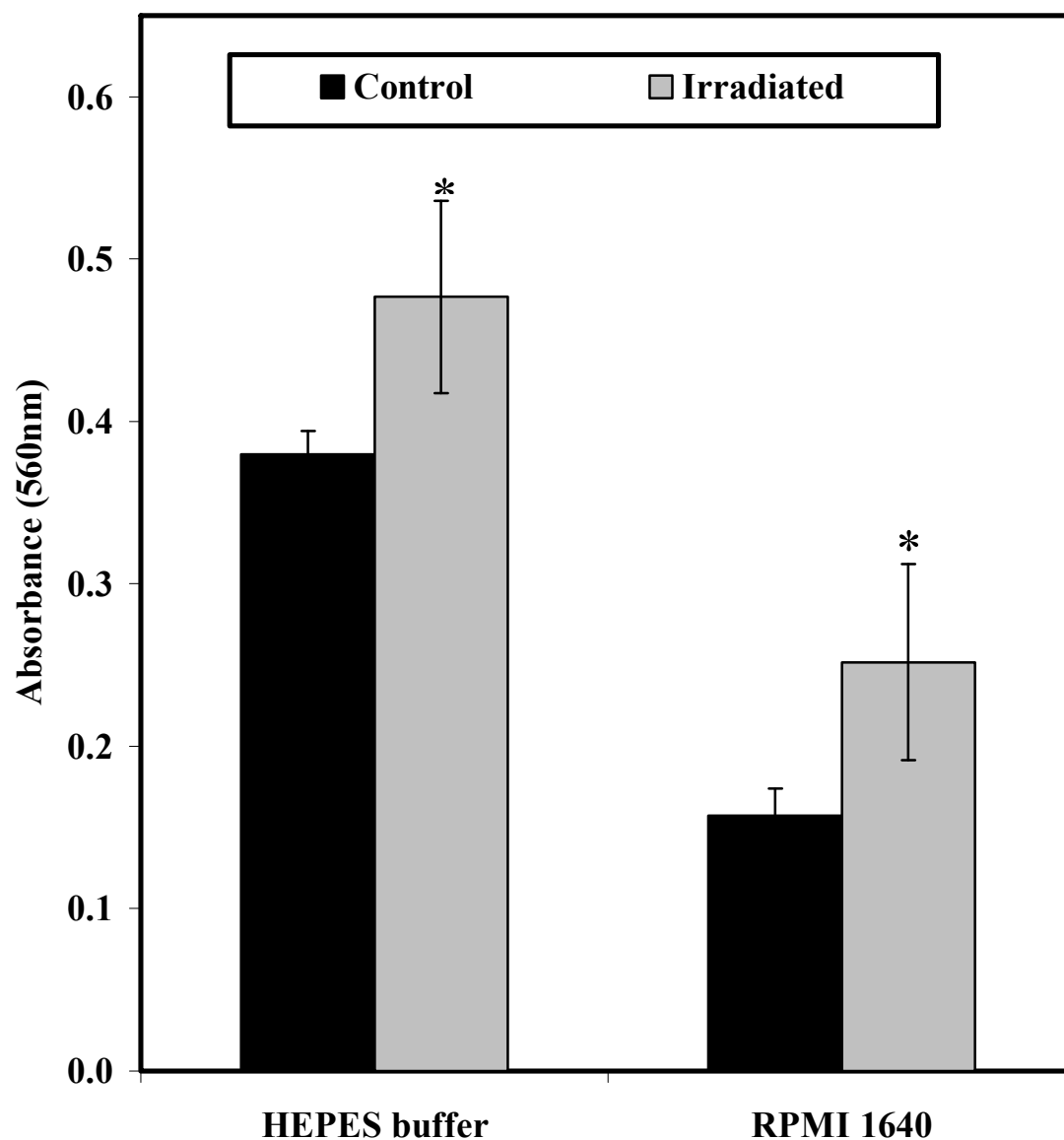
Figure 3.32 Effect of irradiation in different medium systems on the formation of protein hydroperoxides

Protein peroxide concentrations in 2.5×10^6 cells/ml were measured by G-PCA-FOX assay and experimental details are in Section 3.8.2.2.2. The data points were derived from five experiments. * Indicates statistically significant difference between control and irradiated samples at $p < 0.05$ level.

3.8.2.3 Effect of different irradiation times in HEPES buffer and in RPMI-1640 on the formation of protein hydroperoxides

Exponentially growing HL-60 cells were harvested from growth medium and suspended in the HEPES buffer or RPMI-1640 medium at a concentration of 2.5×10^6 cells/ml. The cells were then irradiated for 0, 5, 10, 15 and 20 min (dose rate: 45 Gy/min) at room temperature. After irradiation, the cells were washed with PBS before the G-PCA-FOX assay.

The amounts of protein hydroperoxides increased as the radiation time increased in both media (Fig. 3.33) and there was no significant difference in HEPES buffer and RPMI-1640 medium. The rate of peroxide formation in both media was significantly lower during the initial 15-min irradiation. After 20-min irradiation, peroxide levels were about 3 fold higher than at other irradiation times. This result might due to the depletion of intrinsic antioxidant capacity of the protein. For later experiments, 20 min-irradiation time was used for peroxide studies.
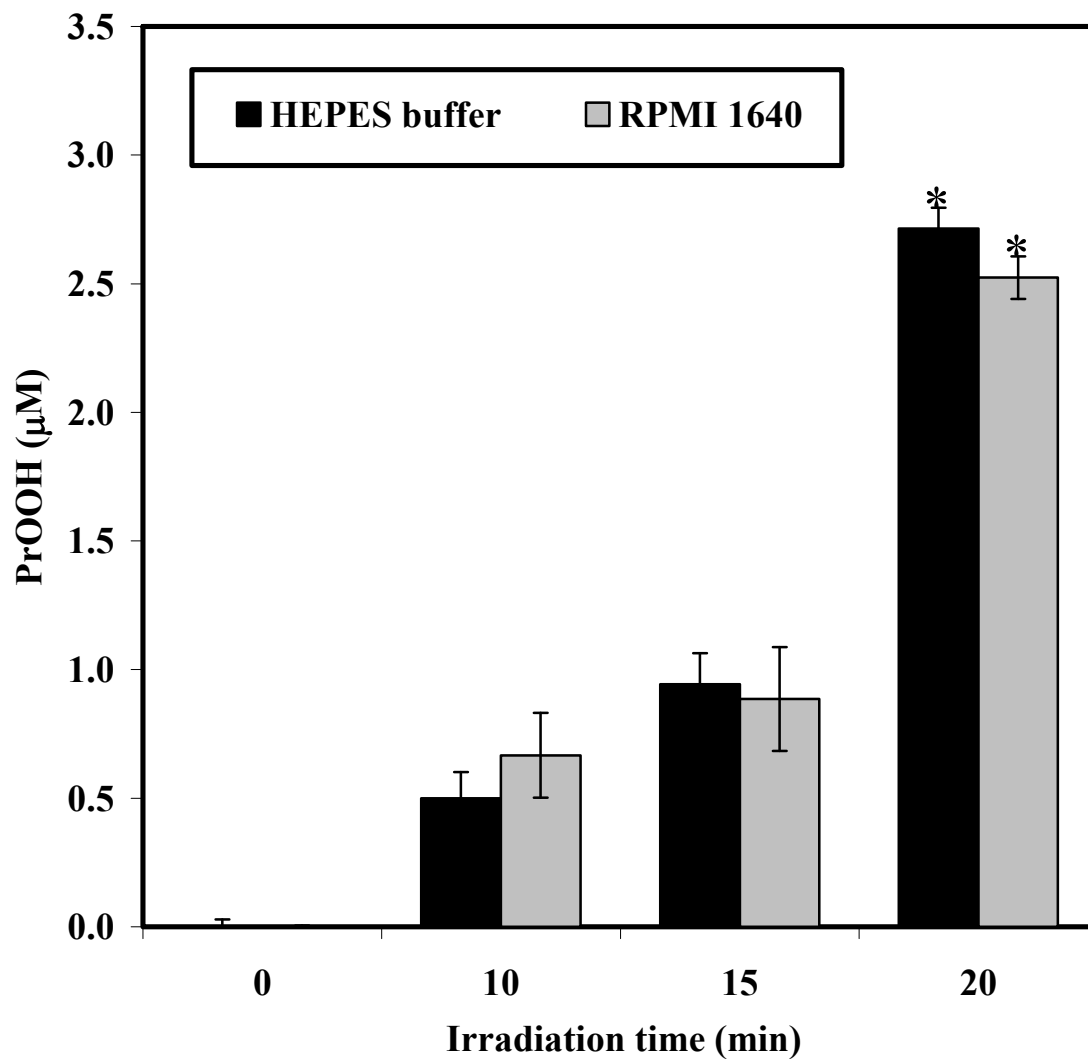
Figure 3.33   Effect of different irradiation times in HEPES buffer and in RPMI-1640 on the formation of protein hydroperoxides

Protein peroxide concentrations of cells were measured by G-PCA-FOX assay and experimental details are in Section 3.8.2.3. The data points were derived from five experiments. * indicates statistically significant difference between control and irradiated samples at $p < 0.05$ level.

3.9 Effect of GSH depletion and repletion on protein peroxide formation in HL-60 cells

Glutathione is the major cellular antioxidant, found in most plants, microorganism and all mammalian tissues. The study of the role of GSH in oxidative stress usually is done by manipulating the GSH levels. A useful approach to understanding the functions of GSH is to remove or enhance its amount and to determine the consequences of these manipulations.

L-Buthionine sulfoximine (BSO) has been the commonly used tool in glutathione research. In the presence of Mg-ATP, BSO (in phosphorylated form) binds tightly to the active site of γ-glutamylcysteine synthetase (γ-GCS), thus irreversibly inhibiting the binding of its neutral substrate L-γ-glutamylphosphate and L-α-aminobutyrate. Low level of GSH can be achieved by inhibiting re-synthesis of GSH when there is an ongoing utilization. After BSO treatment, cellular GSH levels decrease because export of GSH continues in the absence of significant intracellular synthesis (Meister 1994; Shang, Lu et al. 2003).

N-acetylcysteine (NAC), the acetylated variant of the amino acid L-cysteine, is an excellent source of sulfhydryl (SH) groups, and is converted in the body into metabolites capable of stimulating glutathione (GSH) synthesis (Anderson 1997). The sulfhydryl (SH) group is responsible for a great deal of the metabolic activity of NAC, while the acetyl-substituted amino group makes the molecule more stable against oxidation. NAC is able to either reduce extracellular cystine to cysteine, or to be a source of SH metabolites. As a source of SH groups, NAC can stimulate GSH synthesis, enhance glutathione-S-transferase activity,

promote detoxification, and act directly on reactive oxidant radicals (Delneste, Jeannin et al. 1997; Oikawa, Yamada et al. 1999; Schneider, Delles et al. 2005).

Previous studies both *in vitro* and *in vivo* indicate that NAC is able to enhance the intracellular biosynthesis of GSH. In cell culture experiments, NAC promotes the uptake of cysteine from the culture medium for cellular GSH biosynthesis. In vivo, NAC can increase intracellular GSH levels in erythrocytes and in liver and lung cells, and replenish GSH stores following experimental depletion. Experimental results suggest that NAC exerts a protective effect against paraquat-induced cytotoxicity by acting as a GSH precursor and by enhancing intracellular concentrations of GSH (Lin, Xue et al. 1997; Dringen and Hamprecht 1999; Grinberg, Fibach et al. 2005).

3.9.1 The study of intracellular GSH in HL-60 cells

3.9.1.1 Ellman's assay

Total thiols can be measured by loss of absorbance at 412 nm of the solution after incubation with 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB or Ellman's reagent). This reaction must be carried out at pH $\geq$ 8 because DTNB reacts with the sulfur anion of glutathione (pK = 9.2) to liberate one mole of p-nitrothiobenzoate anion per mole of thiol. The nitrothiophenolate anion absorbs light at 412 nm, has a molar absorption coefficient ε = 1.41×10^4 $M^{-1}cm^{-1}$ (0.10 M phosphate buffer + 0.01 M EDTA) (Duggan, Rait et al. 2002) and provides therefore a stoichiometric measure of the thiol concentration.

3.9.1.2 GSH standard curve in Ellman's assay

The GSH solution was prepared immediately in 3 % PCA before use, and the GSH concentration was in the range of 1-10 μM. At pH 8.0 at room temperature, the reaction between 10 mM DTNB and GSH was complete within 2 minutes at most. The absorbance at 412 nm was recorded (Ellman 1959; Moss and Swarup 1988). The standard curve was generated by plotting A_{412} against the concentration of GSH (Fig. 3.34). The calculated molar absorption coefficient was 1.52×10^4 mol^{-1}cm^{-1}.

3.9.1.3 GSH concentration in different cell numbers

Ellman's assay measures the sums total of cellular thiols, GSH and protein thiols. The addition of PCA to cells precipitated thiol proteins and removes the cellular proteins from the solution. Finally, only intracellular GSH in the cellular solution was measured in Ellman's assay in these experiments (Sies 1999).

Different numbers of HL-60 cells were harvested by centrifugation, and the cell pellets were treated with 100 μl of 3 % PCA, left on ice for 10 minutes and then centrifuged for 16,500 rpm at 10 minutes. The supernatants were mixed with 50 μl 0.5 M Na_3PO_4, 750 μl 0.1 M NaH_2PO_4/ 5 mM EDTA pH 7.5 and 100 μl of 10 mM DTNB. Using the molar absorption coefficient from the previous standard curve, GSH concentration in the cells was calculated. Concentrations (M) were converted to amounts (mole) by using volumes of solutions or cells used (Fig. 3.35). The cell number used in following experiment was 2.0×10^6 cells which had intracellular GSH at 6.6 ± 0.15 nmole. The results of Ellman's assay indicated the cellular thiols; we assumed that GSH is the dominate thiol group in cells.
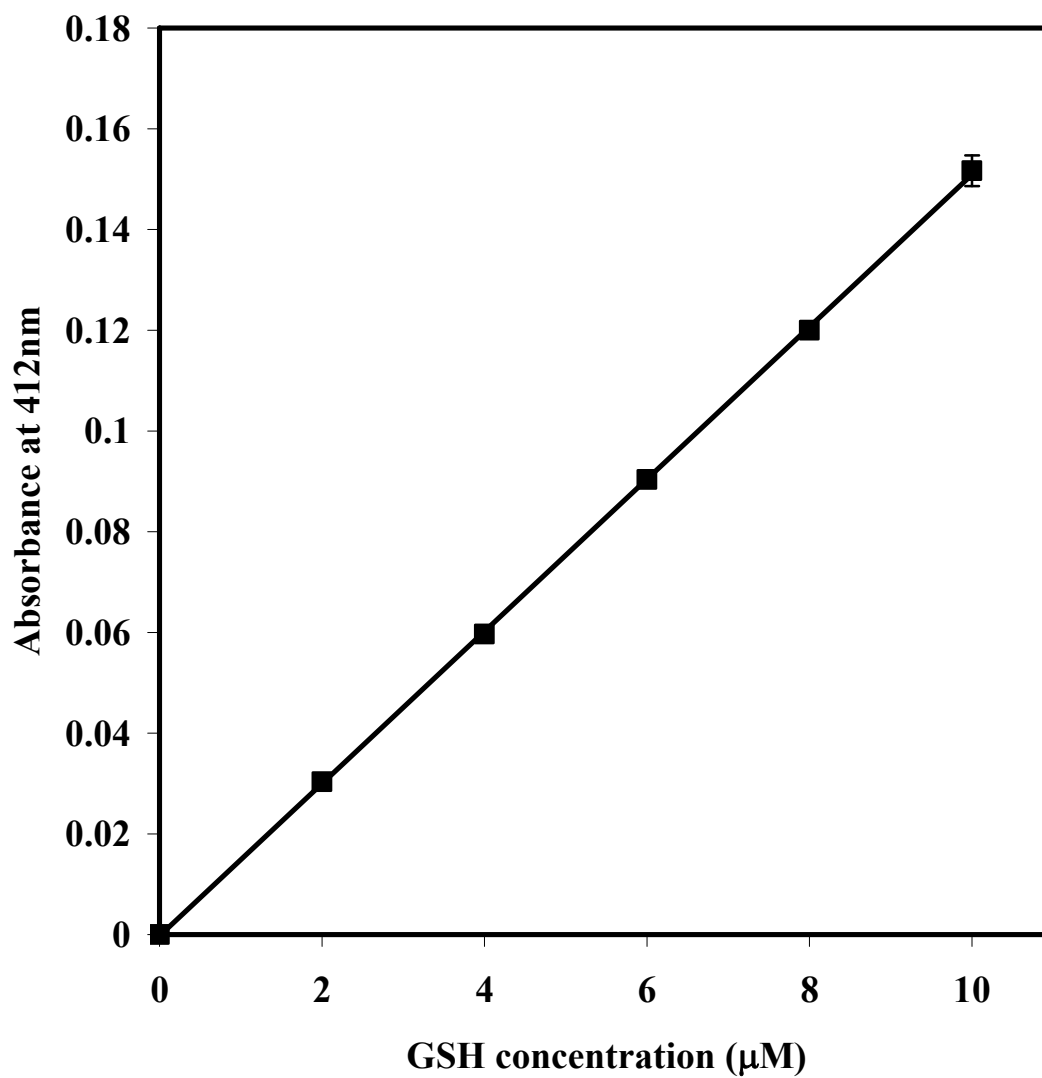
Figure 3.34 The standard curve for reaction between GSH and DTNB (Ellman's reagent)

GSH standard curve in Ellman's assay was performed *in vitro*. Experimental details are in Section 3.9.1.2.Each data point represents the mean ± SD of three values.
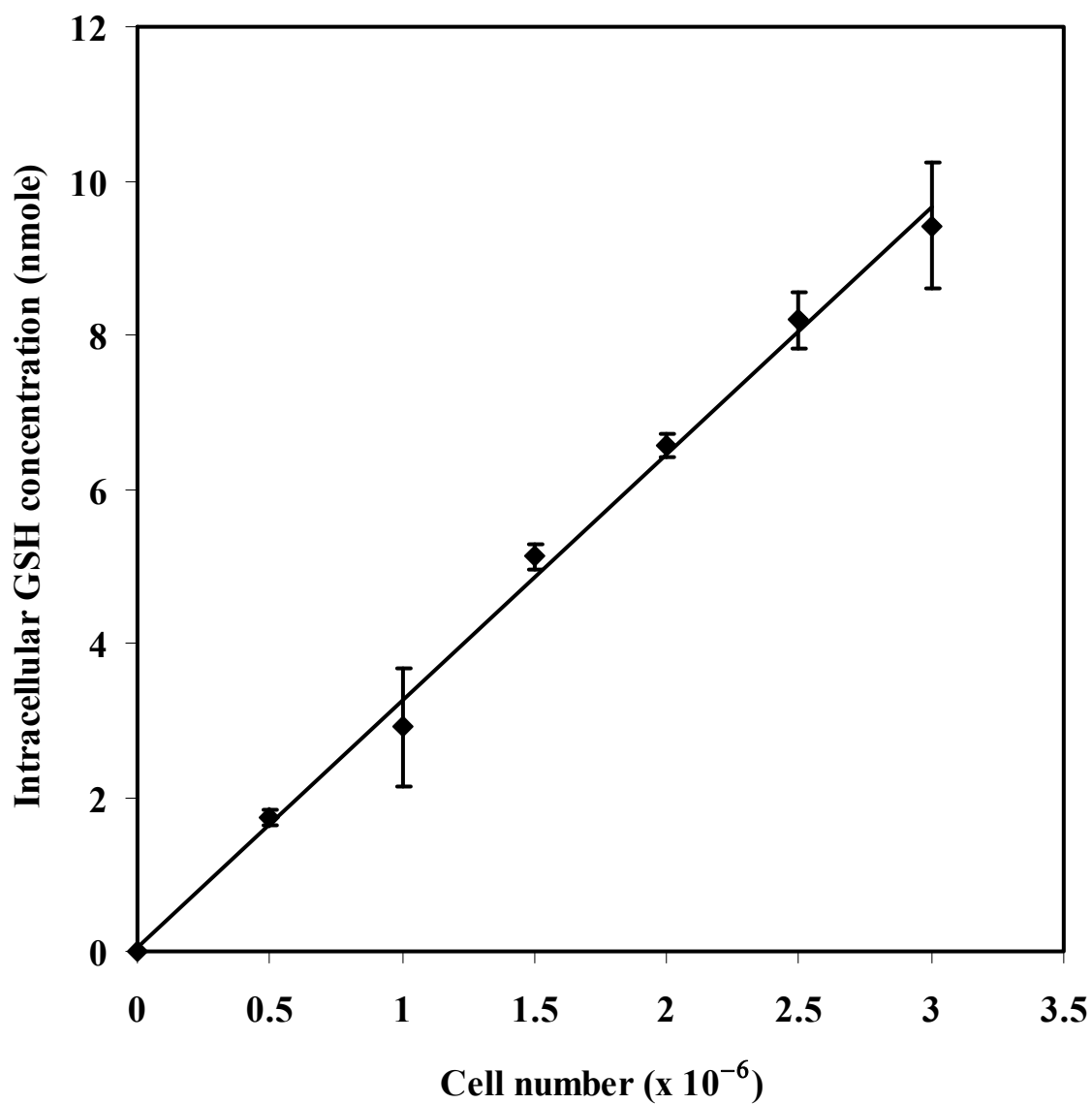
Figure 3.35 The intracellular GSH concentration in different cell numbers of HL-60 cells

Experimental details are in Section 3.9.1.3. Each data point in the curve represents the mean ± SD of three measurements.

3.9.2 Protein estimate in HL-60 cells

3.9.2.1 The standard curve for reaction between BSA and Bradford reagent

BSA solutions containing from 1 to 50 μg protein were diluted from 10 mg/ml BSA stock solution, 20 μl of each BSA solution was then mixed with 1 ml BioRad Dye Protein Reagent. After a short incubation, A_{595} was measured in a spectrophotometer. BSA standard curve was generated by plotting A_{595} against the concentration of BSA. The protein standard curve was generated by plotting change of A_{595} against the concentration of BSA (Fig. 3.36). The molar absorption coefficient was $\varepsilon = 3.0 \times 10^4$ (ml μg)$^{-1}$cm^{-1}.

3.9.2.2 Total protein concentration in different cell numbers

The different cell numbers of HL-60 cells were harvested by centrifugation, and the cell pellets were solubilized in 0.3 N NaOH. Cell protein was determined by the Bradford assay. The curve was generated by plotting cell numbers against the amount of total protein. Using the molar coefficient gained in previous standard curve (BSA), protein amounts in different cell numbers can be calculated (Fig.3.37). The cell number used in following experiment was 2.0×10^6 cells which had 184.9 ± 5.5 μg. of intracellular protein. At the high protein amounts, the absorbance was not linearly-dependent on the cell numbers. This is due to the Bradford's assay limitation at high protein concentrations.
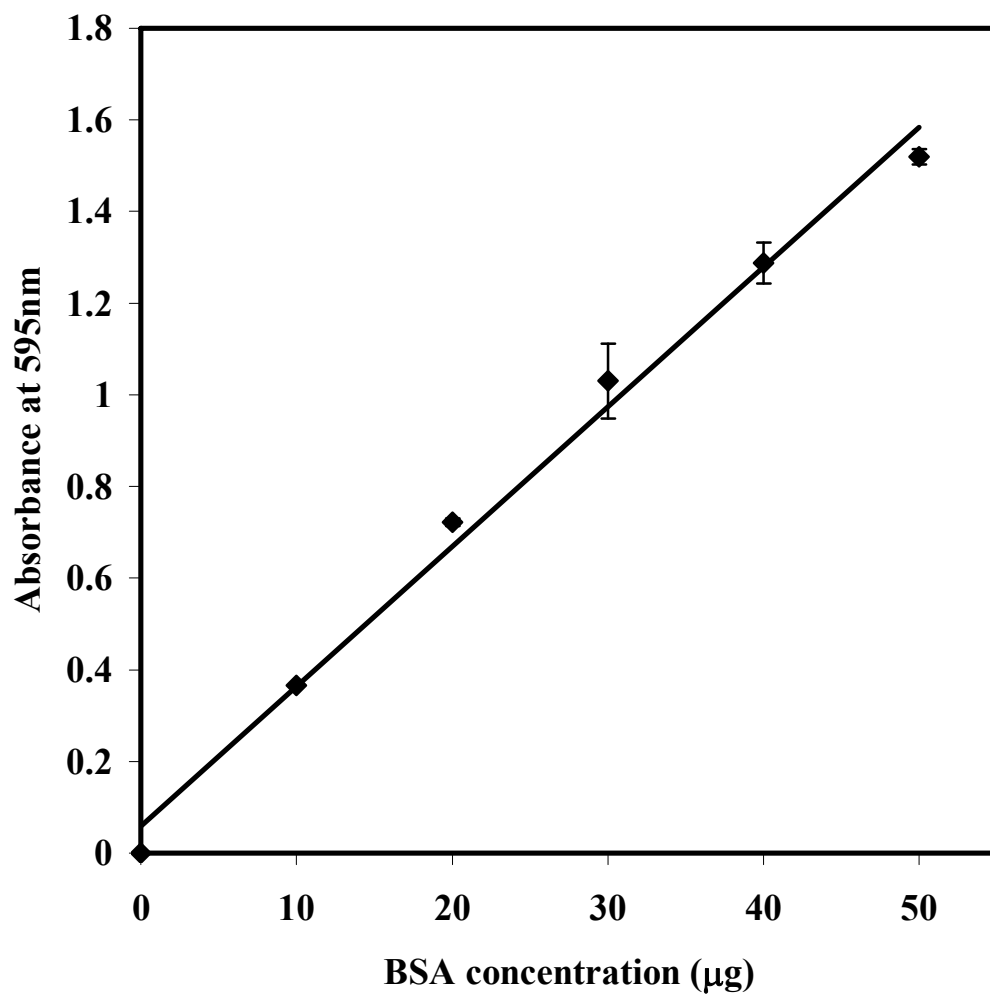
Figure 3.36 The standard curve for reaction between BSA and Bradford reagent

The BSA solution was used to make the standard curve of protein amount in Bradford protein assay and experimental details are in Section 3.9.2.1. The data points were derived from three experiments, with some error bars obscured by the symbols.
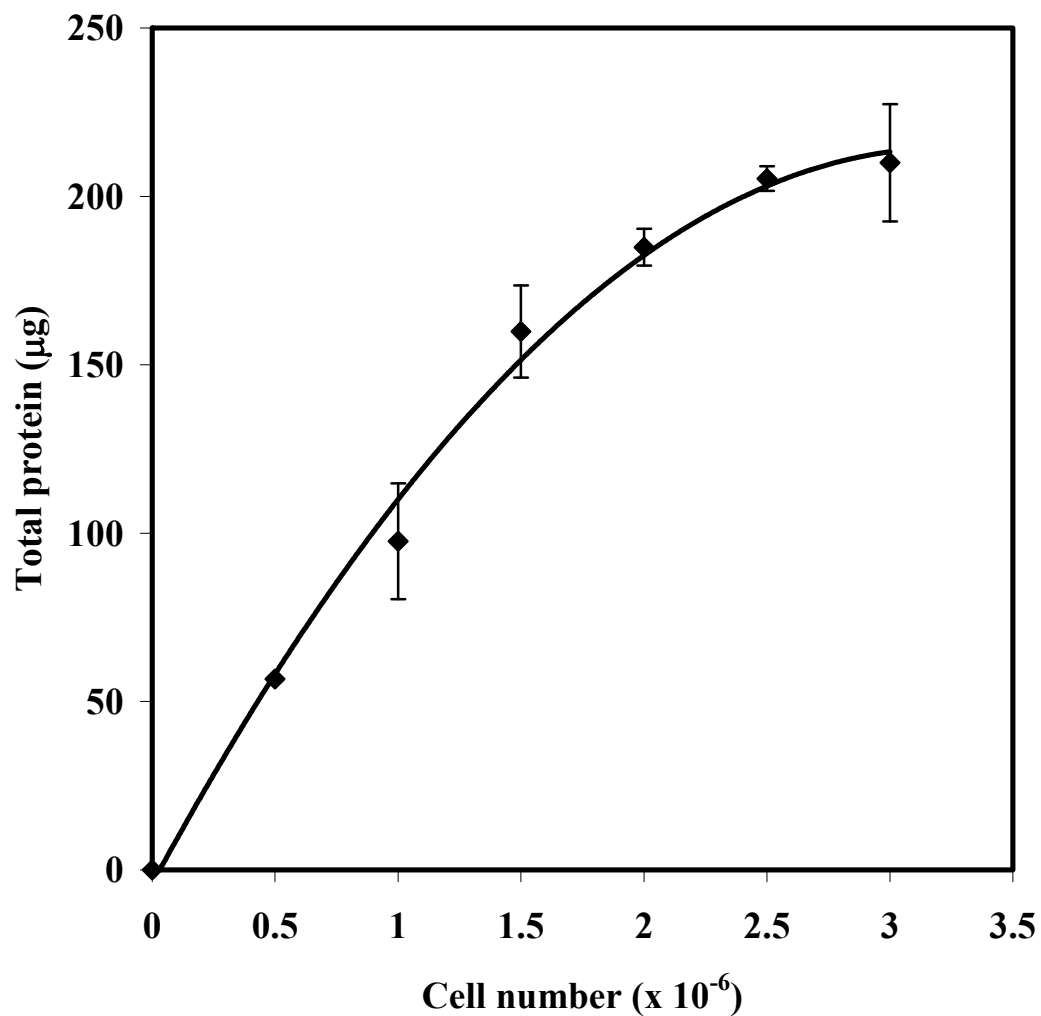
Figure 3.37 The total intracellular protein amount of HL-60 cells in different cell numbers

The intracellular protein amount of HL-60 cells in different cell numbers were also measured in Bradford protein assay and experimental details are in Section 3.9.2.2. Each data point represents the mean ± SD of three values.

3.9.3 Effect of γ irradiation on intracellular GSH levels

Exponentially growing HL-60 cells were harvested from RPMI-1640 growth medium and suspended in serum-free RPMI-1640 medium at a concentration of ~2× 10^6 cells/ml. The cell suspensions were irradiated at room temperature for 0, 5, 10, 15, and 20 min with a dose rate of 45 Gy/min. At each time point, triplicate 1ml cultures were harvested in glass tubes and stored on ice before GSH measurements, which wer carried out as described in section 3.9.1.3. The results were expressed as nmole GSH per mg total protein. After 5 min irradiation, GSH level dropped by 2.6 % compared to zero time (Fig. 3.38). The GSH level decreased by 12.2 % after 10 min irradiation, 25.9 % after 15 min, and 34.9 % after 20 min irradiation.
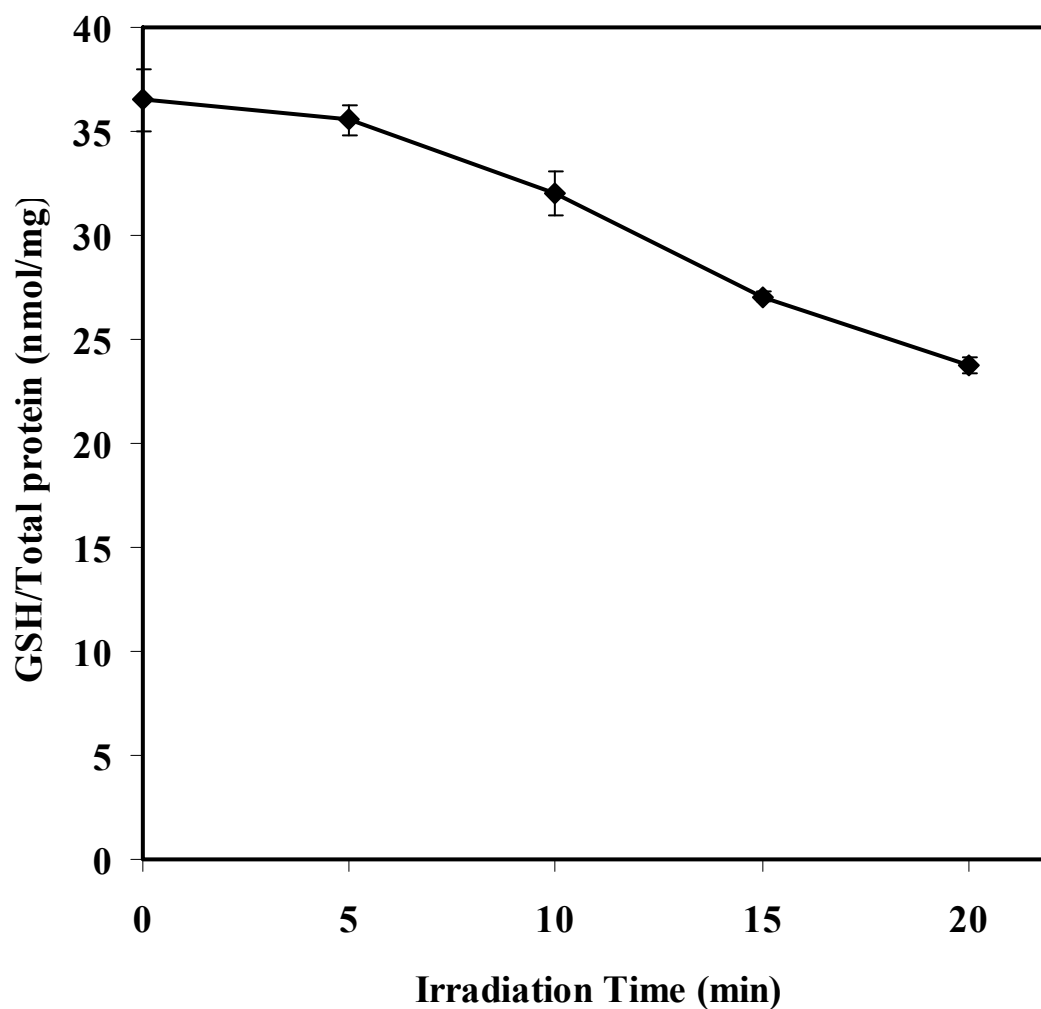
Figure 3.38 Effect of irradiation on intracellular GSH levels

Intracellular GSH was measured by Ellman's assay and total protein was determined by Bradford's assay. Experimental details are in Section 3.9.3. The results were expressed an nmole GSH per mg total protein. The data points were derived from triplicate experiments, with error bars partly obscured by the symbols.

3.9.4 Effect of BSO on HL-60 cells

3.9.4.1 The effect of BSO on intracellular GSH levels

HL-60 cells (~1×10^6 cells/ml) were incubated with 0, 50, 100, 150, 200, 250, 300, and 500 μM BSO in RPMI-1640 growth medium at 37 °C for 24 h, triplicate 1ml cultures were harvested and washed with PBS twice. The cell pellets were lysed with 100 μl of 3 % PCA (ice cold). After centrifugation, the supernatant was transferred to Eppendorf tubes, and the pH was adjusted to ~8.0. The GSH levels were measured according to Ellman's assay and protein by the Bradford method, so that results were expressed as nmole GSH per mg total protein.

As shown in Figure 3.39, BSO treatment of the cells caused significant decrease of intracellular GSH. 50 μM BSO treatment for 24 h caused 78.1 % depletion of GSH level (8.1 nmol/mg protein compared to control 36.9 nmol/mg protein). As the concentration of BSO increased, the total intracellular GSH level further slightly decreased, 100 μM BSO giving 85.7 % depletion (5.3 nmol/mg protein), and 200 μM BSO giving 85.1 % depletion (5.5 nmol/mg protein). For subsequent experiments, 100 μM BSO was used, which gave over 85 % GSH depletion of HL-60 cells.
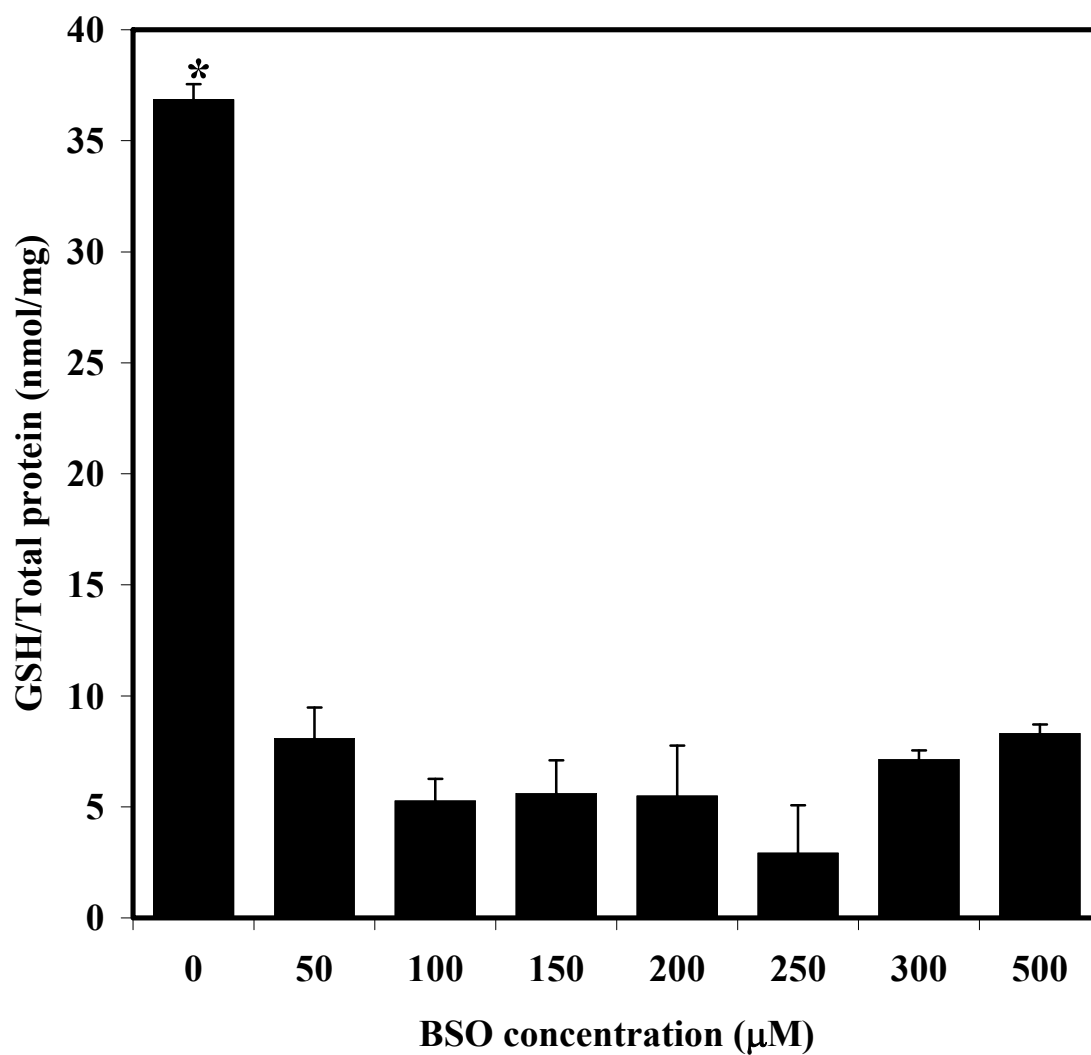
Figure 3.39 Effect of BSO on GSH levels of HL-60 cells

The GSH of cells treated with different concentrations of BSO was measured by Ellman's assay and the results were expressed as nanomoles GSH per mg of cell protein. Experimental details are in Section 3.9.4. Each bar represents means of triplicate experiments. *: indicates statistically significant difference between control and BSO-treated samples at $p<0.05$ level.

3.9.4.2 Effect of BSO on cell viability

HL-60 cells (~1 × 10^6 cells/ml) were incubated with 0, 50, 100, 150, 200, 250, 300, and 500 μM BSO in RPMI-1640 growth medium at 37 ^{o}C for 24 h. After incubation, the cell were washed with PBS twice and 200 μl of cells (n=5) were seeded in 96-well plate for MTT assay. BSO treatment has no effect on cell viability (Fig. 3.40). The cell viability was maintained at about 100 % in all different BSO concentrations. For following experiments, 100 μM BSO was used.

3.9.4.3 Effect of BSO on intracellular GSH levels in γ irradiated cells

HL-60 cells were suspended in RPMI-1640 growth medium at a concentration of ~1 × 10^6 cells/ml and incubated with 100 μM BSO or equal volumes of RPMI-1640 at 37 ^{o}C for 24 h. After incubation, the cells were harvested, suspended in serum-free RPMI-1640 medium (2 × 10^6 cells/ml) and irradiated in 60-mm Petri dishes at room temperature for 0, 5, 10, 15, and 20 min with a dose rate of 45 Gy/min. At each time point, triplicate 1ml cultures were harvested in glass tubes and stored on ice before GSH measurement. The results were expressed as nmole GSH per mg total protein. BSO treatment for 24 h caused 75-84 % depletion of GSH level of HL-60 cells at all different irradiation times (Fig. 3.41). The amounts of intracellular GSH in BSO- treated cells were significantly decreased with increasing irradiation time.
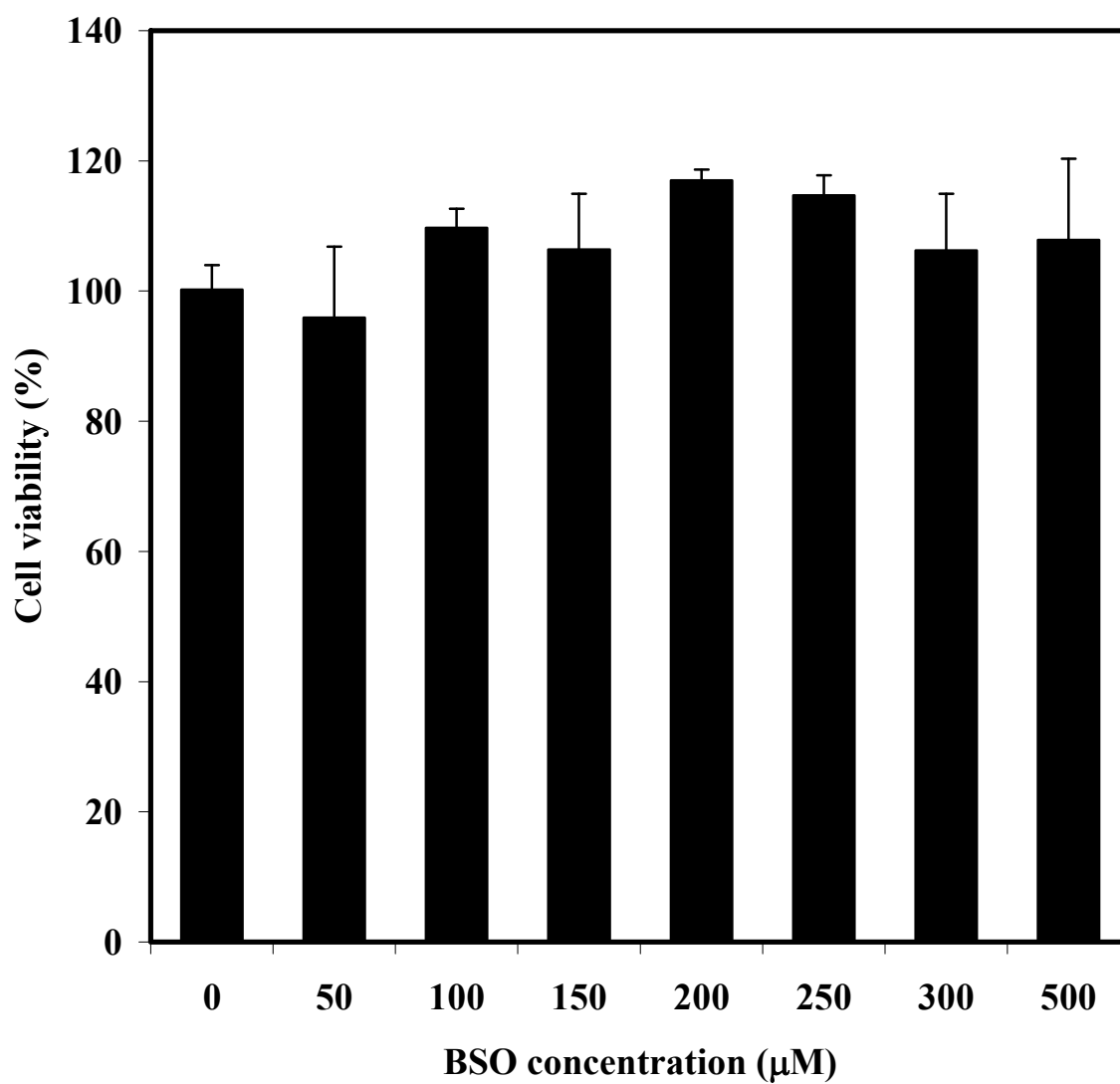
Figure 3.40 Effect of BSO on cell viability

The cell viability was measured by MTT assay. Experimental details are in Section 3.9.4.2. The data points were derived from triplicate experiments.
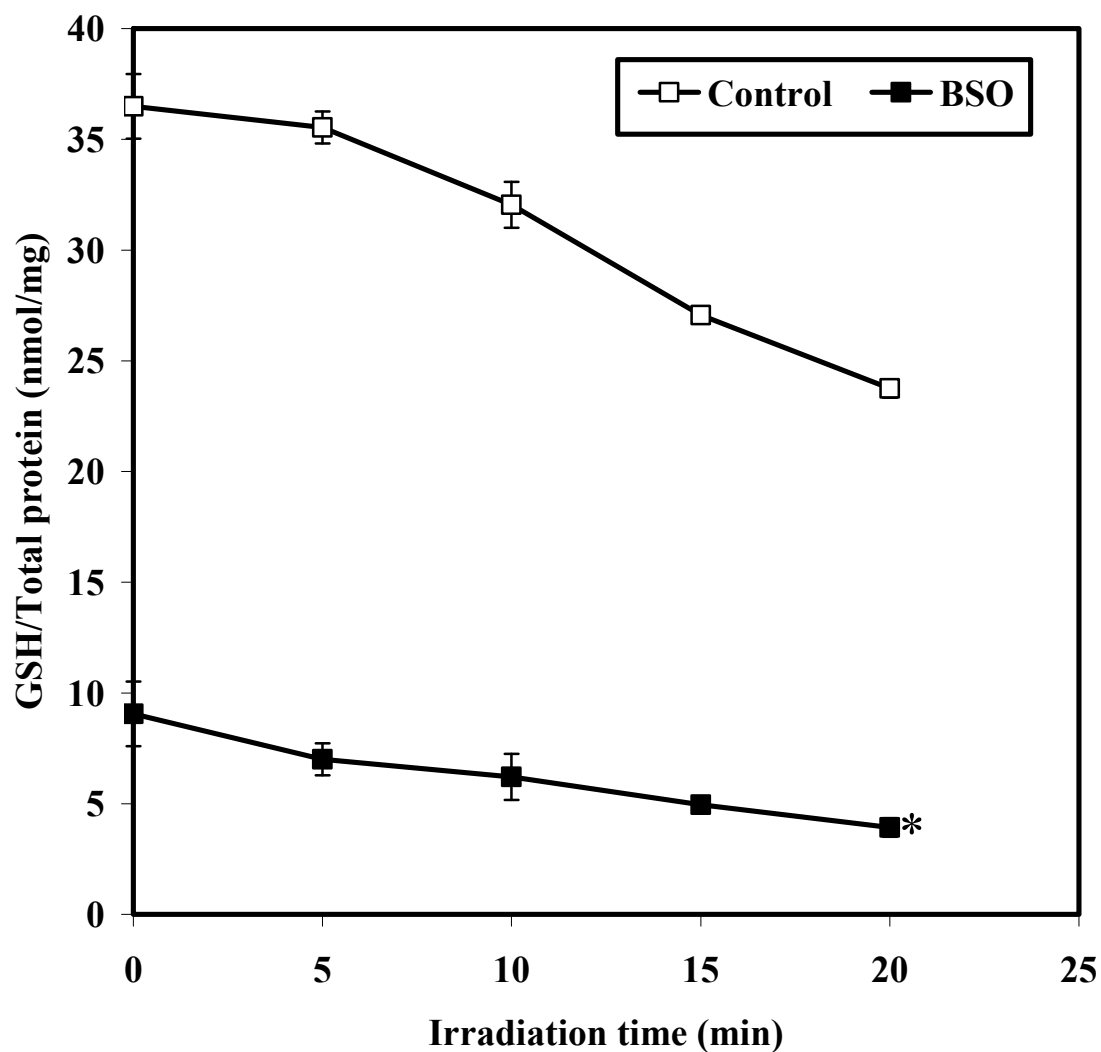
Figure 3.41 Effect of BSO on intracellular GSH levels in irradiated cells

Intracellular GSH was measured by Ellman's assay and total protein was determined by Bradford's assay. Experimental details are in Section 3.9.4.3. Each bar represents the mean ± SD of three experiments. *: indicates statistically significant difference between BSO-treated and control at $p<0.05$ level.

3.9.4.4 *Effect of GSH depletion on protein hydroperoxide formation in γ irradiated cells*

Exponentially growing HL-60 cells were harvested from RPMI-1640 growth medium and suspended in serum-free RPMI-1640 medium at a concentration of ~2 × 10^6 cells/ml. The cell suspensions were irradiated at room temperature for 0, 5, 10, 15, and 20 min with a dose rate of 45 Gy/min. After irradiation, the cells were washed with PBS before G-PCA-FOX assay. After irradiation, GSH depleted cells had a significant increase in the amounts of protein peroxides compared to non-depleted cells in 10, 15, and 20 min irradiated cells

The results are shown in Figure 3.42 and Table 3.21. The results revealed that GSH depletion compromised the ability of cells to inhibit the formation or detoxify protein peroxides. As described before, 20 min irradiation alone depleted normal GSH level by about 60 % (Fig. 3.38). BSO-treated cells had virtually no GSH and showed about 130 % increase in the levels of protein peroxides generated by 20 min irradiation. The quantitative correspondence between the loss of GSH and formation of protein peroxides suggested that GSH was instrumental in inhibition of formation or decomposition of protein peroxides in HL-60 cells. Furthermore, the rate of peroxide formation was lower in the initial 15-min irradiation in the GSH depleted cells. After 20-min irradiation, peroxide levels were 2.3 fold higher than at other irradiation times. This phenomenon was caused by the presence of some GSH in the first fifteen minutes of irradiation which helped to detoxify protein peroxides.

Table 3.21 Hydroperoxide yields in 20 min irradiated cells in the presence or absence of BSO

PrOOH (µM)	ΔA_{560}	PrOOH (µM)
No-BSO	0.082 ± 0.003	2.52 ± 0.08
BSO	$*0.205 \pm 0.001$	$*5.86 \pm 0.02$

* Indicates statistically significant difference to the BSO-treated and control cells at *p*<0.05 level. PrOOH: protein peroxides.
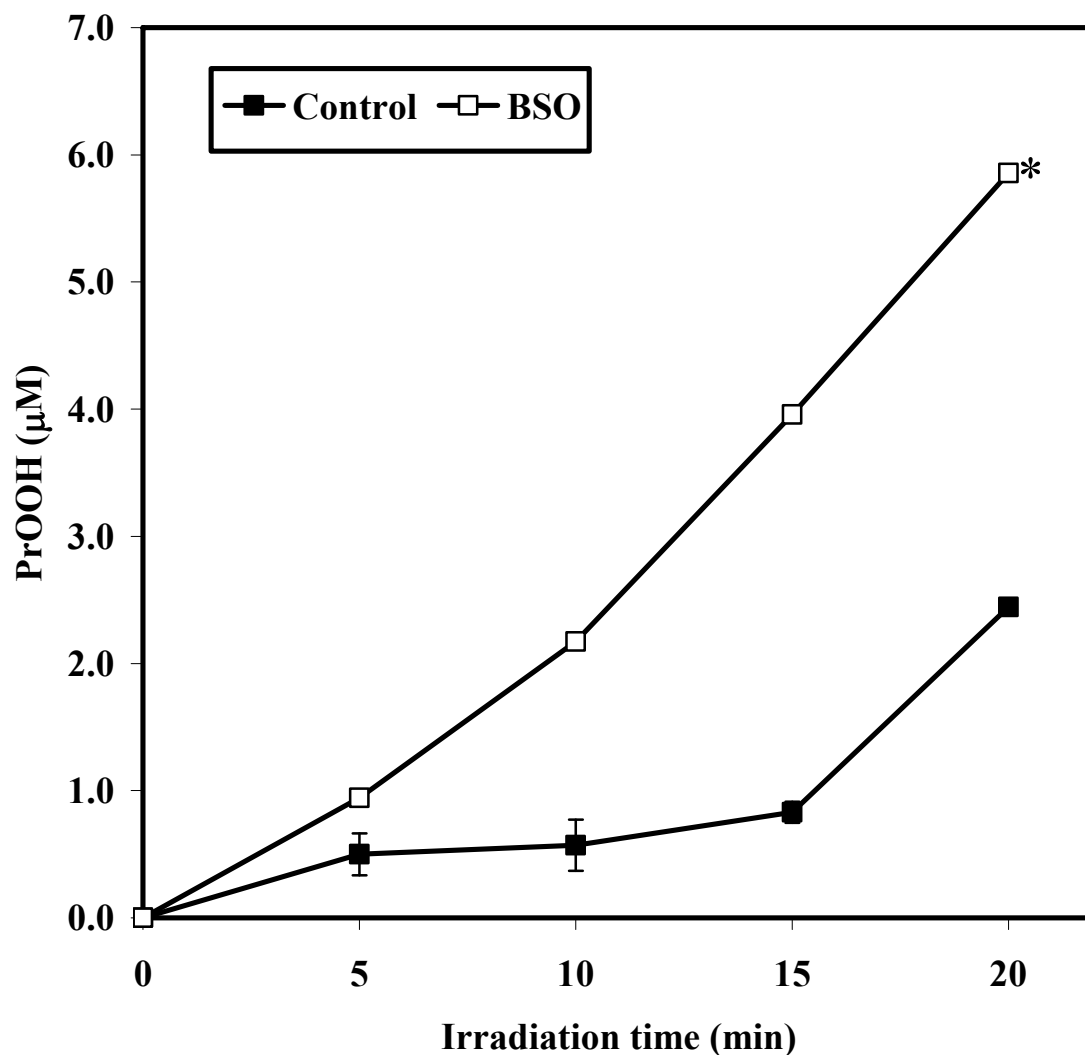
Figure 3.42 Effect of GSH depletion on protein hydroperoxides formation in irradiated cells

The protein hydroperoxide concentrations were measured by G-PCA-FOX assay. Experimental details are in Section 3.9.4.5. The data points were derived from five experiments. *: indicates statistically significant difference between control and irradiated samples at $p<0.05$ level.

3.9.5 Effect of NAC on HL-60 cells

3.9.5.1 The effect of NAC on intracellular GSH levels

HL-60 cells ($\sim 2 \times 10^6$ cells/ml) were incubated with 0, 1, 5 10, 15 and 20 mM NAC in rerum-free RPMI-1640 medium at 37 $^{\circ}$C for 3 h, triplicate 1ml cultures were harvested and washed with PBS twice. The cell pellets were lysed with 100μl of 3 % PCA (ice cold). After centrifugation, the supernatant was transferred to Eppendorf tubes, pH was adjusted to ~8.0. The GSH levels were measured according to Ellman's assay and protein by the Bradford method, so that results were expressed as nmole GSH per mg total protein. Any intracellular cysteine would also be measured, but its contribution would be insignificant.

As shown in Figure 3.43, 1 mM NAC treatment for 3 h caused 22 % enhancement of GSH level in HL-60 cells (32.7 nmole/mg protein compared to control 39.9 nmole/mg protein). As the concentration of NAC increased, the total intracellular GSH level dramatically increased, 5 mM NAC giving 79 % enhancement (58.7 nmole/mg protein), and 20 mM NAC giving 310 % enhancement (134.1 nmole/mg protein). For subsequent experiments, 15 mM NAC was used, which gave over 223 % GSH enhancement.
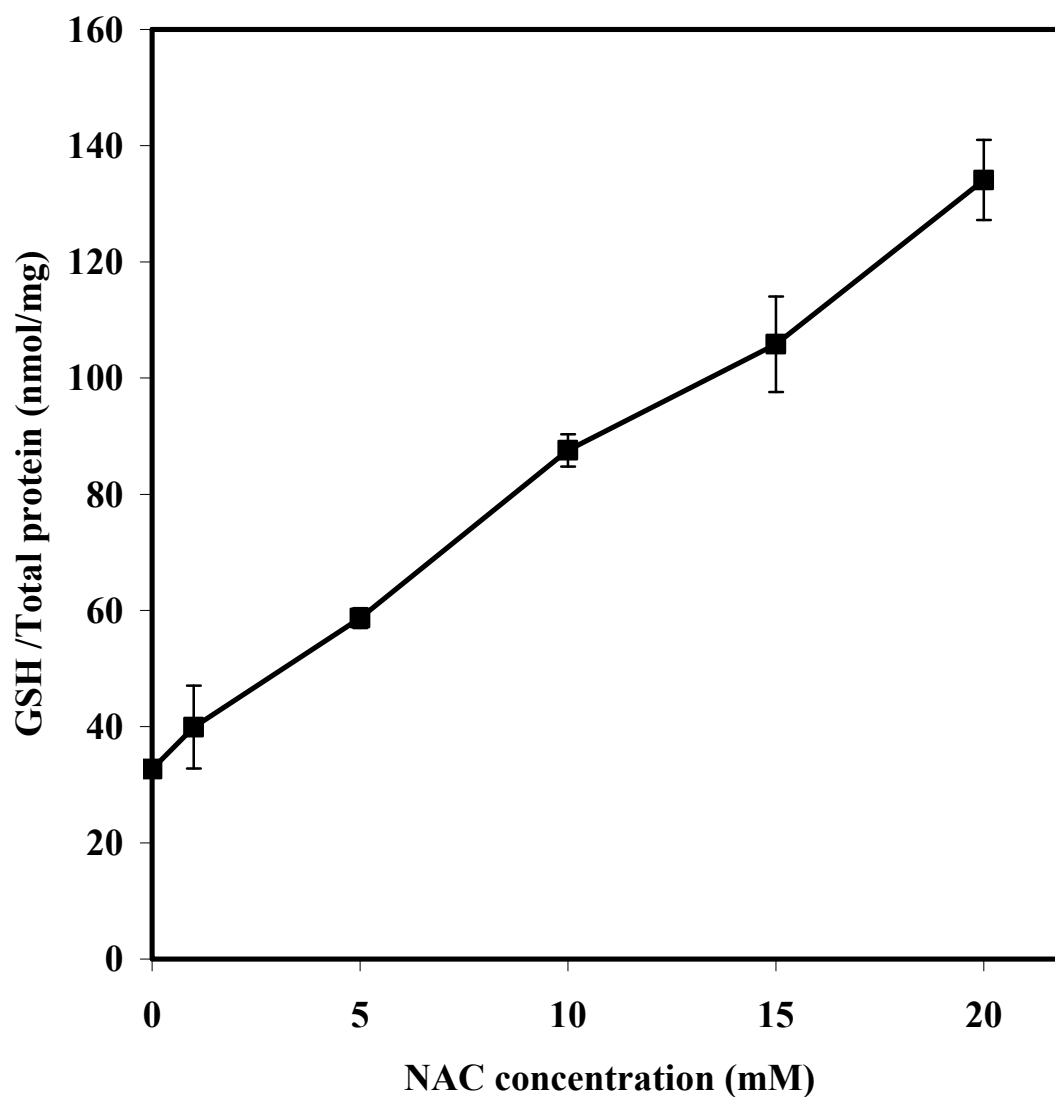
Figure 3.43 Effect of NAC on GSH levels of HL-60 cells

The GSH in cells treated with different concentration of NAC was measured by Ellman's assay, total protein was determined by Bradford's assay and the results were expressed as micromoles GSH per mg of cell protein. Experimental details are in Section 3.9.5.1. Each bar represents means of triplicate experiments.

3.9.5.2 Effect of NAC on cell viability

HL-60 cells ($\sim 1 \times 10^6$ cells/ml) were incubated with 0, 1, 5, 10, 15 and 20 mM NAC in serum-free RPMI-1640 medium at 37 ^{o}C for 3 h. After incubation, the cells were washed with PBS twice and 200µl of cells (n=5) were seeded in 96-well plate for MTT assay. NAC treatment has no effect on cell viability (Fig. 3.44). For following experiments, 15 mM NAC was used.

3.9.5.3 Effect of NAC on intracellular GSH levels in γ irradiated cells

HL-60 cells were suspended in RPMI-1640 serum-free medium at a concentration of $\sim 2 \times 10^6$ cells/ml and incubated with 15 mM NAC or equal volumes of RPMI-1640 at 37 ^{o}C for 3 h. After incubation, the cells were harvested and suspended in serum-free RPMI-1640 medium (2×10^6 cells/ml) and irradiated in 60-mm Petri dishes at room temperature for 0, 5, 10, 15, and 20 min with a dose rate of 45 Gy/min. At each time point, triplicate 1ml cultures were harvested in glass tubes and store on ice before GSH measurement. The results were expressed as nmole GSH per mg total protein. NAC treatment for 3 hours caused over 300 % enhancement of GSH level in HL-60 cells at all irradiation times (Fig. 3.45). The amount of intracellular GSH slightly decreased with the increasing irradiation time in both groups.
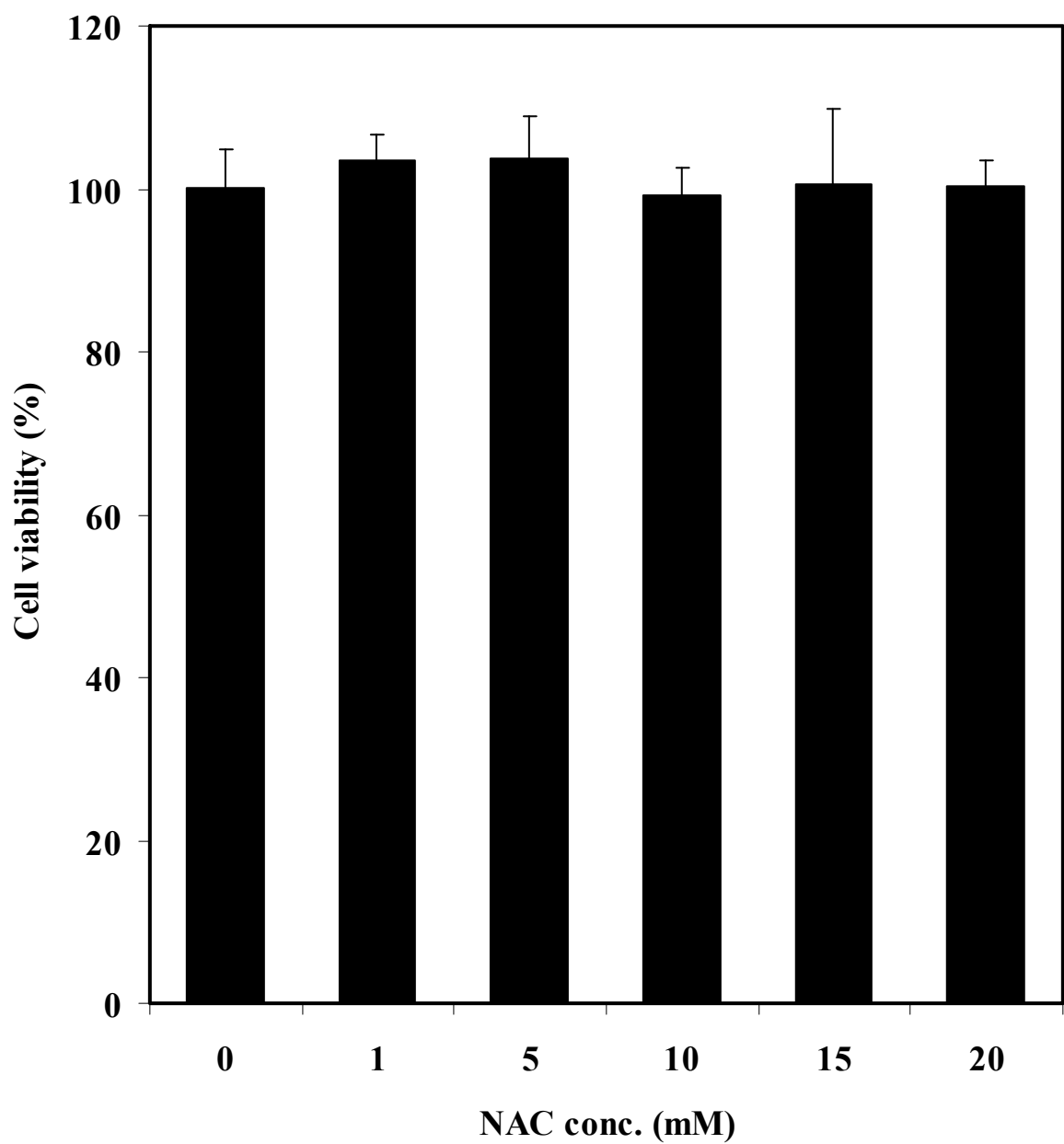
Figure 3.44 Effect of NAC on cell viability

The cell viability was measured by MTT assay. Experimental details are in Section 3.9.5.2. The data points were derived from triplicate experiments, with error bars.
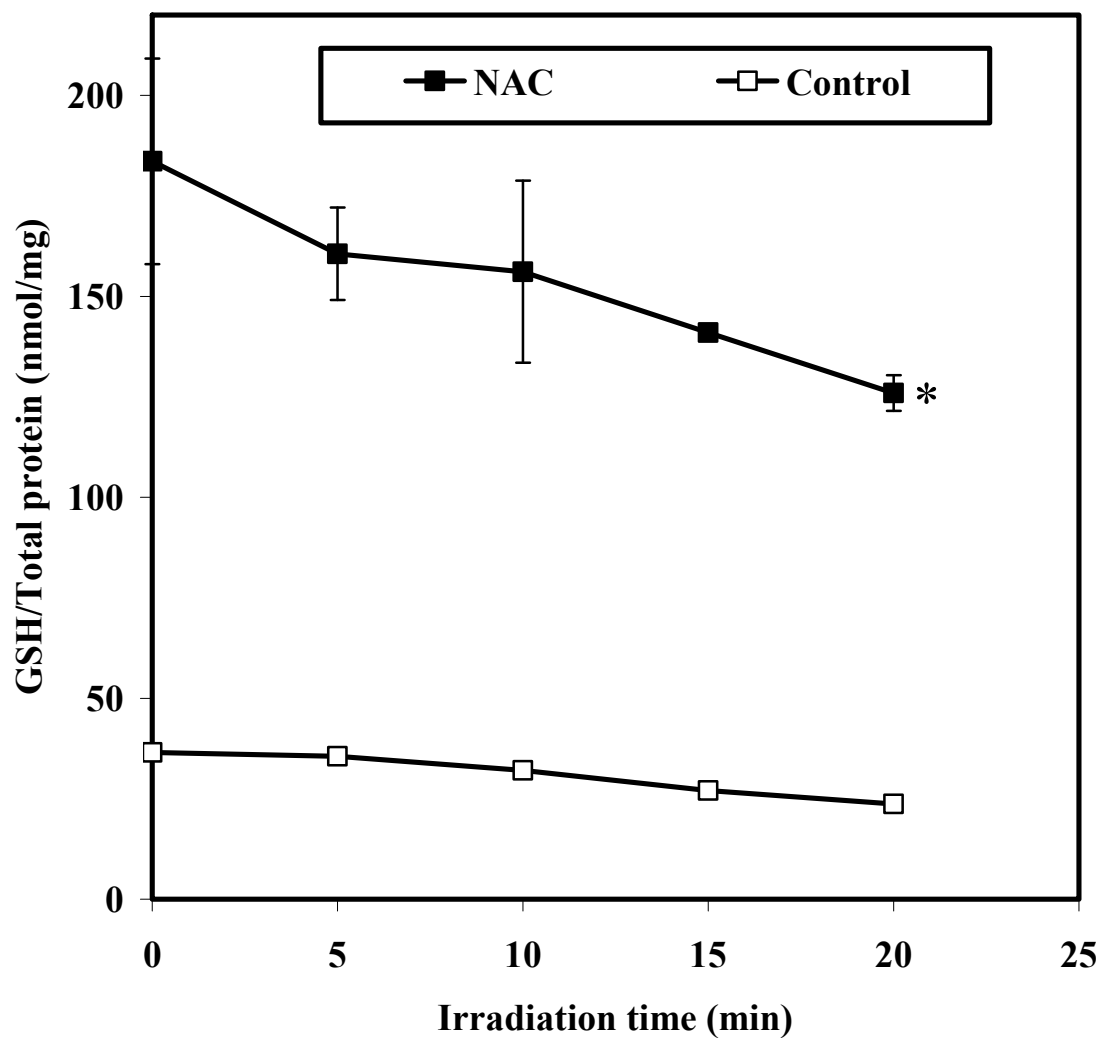
**Figure 3.45 Effect of NAC on intracellular GSH levels in irradiated cells**

Intracellular GSH was measured by Ellman's assay and total protein was determined by Bradford's assay. Experimental details are in Section 3.9.5.3. Each bar represents the mean ± SD of three experiments. *: indicates statistically significant difference between NAC and No-NAC treatment samples at $p<0.05$ level.

3.9.5.4 Effect of GSH enhancement on protein hydroperoxide formation in γ-irradiated cells

Exponentially growing HL-60 cells were harvested from RPMI-1640 growth medium and resuspended in serum-free RPMI-1640 medium at a concentration of ~2 × 10^6 cells/ml. Cells were incubated with 15 mM NAC or equal volumes of RPMI-1640 at 37 °C for 3 h. The cell suspensions were irradiated at room temperature for 0, 5, 10, 15, and 20 min with a dose rate of 45 Gy/min. After irradiation, the cells were washed with PBS before G-PCA-FOX assay.

After irradiation, GSH-enhanced cells had a significant reduction in the amounts of protein peroxides compared to control and irradiated cells. The results in Figure 3.46 and Table 3.22 revealed that GSH loss on irradiation compromised the ability of the cells to inhibit the formation or detoxify protein peroxides and reduced γ irradiation increased oxidative damage. After 20 min irradiation, NAC-treated cells had high GSH levels and showed an 80 % decrease in the levels of protein peroxides compared with control cells.

Table 3.22 Hydroperoxide yields in 20 min-irradiated cells in the presence or absence of NAC

PrOOH (µM)	ΔA_{560}	PrOOH (µM)
No-NAC	0.082 ± 0.003	2.52 ± 0.08
NAC	$*0.018 \pm 0.001$	$*0.51 \pm 0.03$

* Indicates statistically significant difference to the NAC-treated and control cells at $p<0.05$ level. PrOOH: protein peroxides.
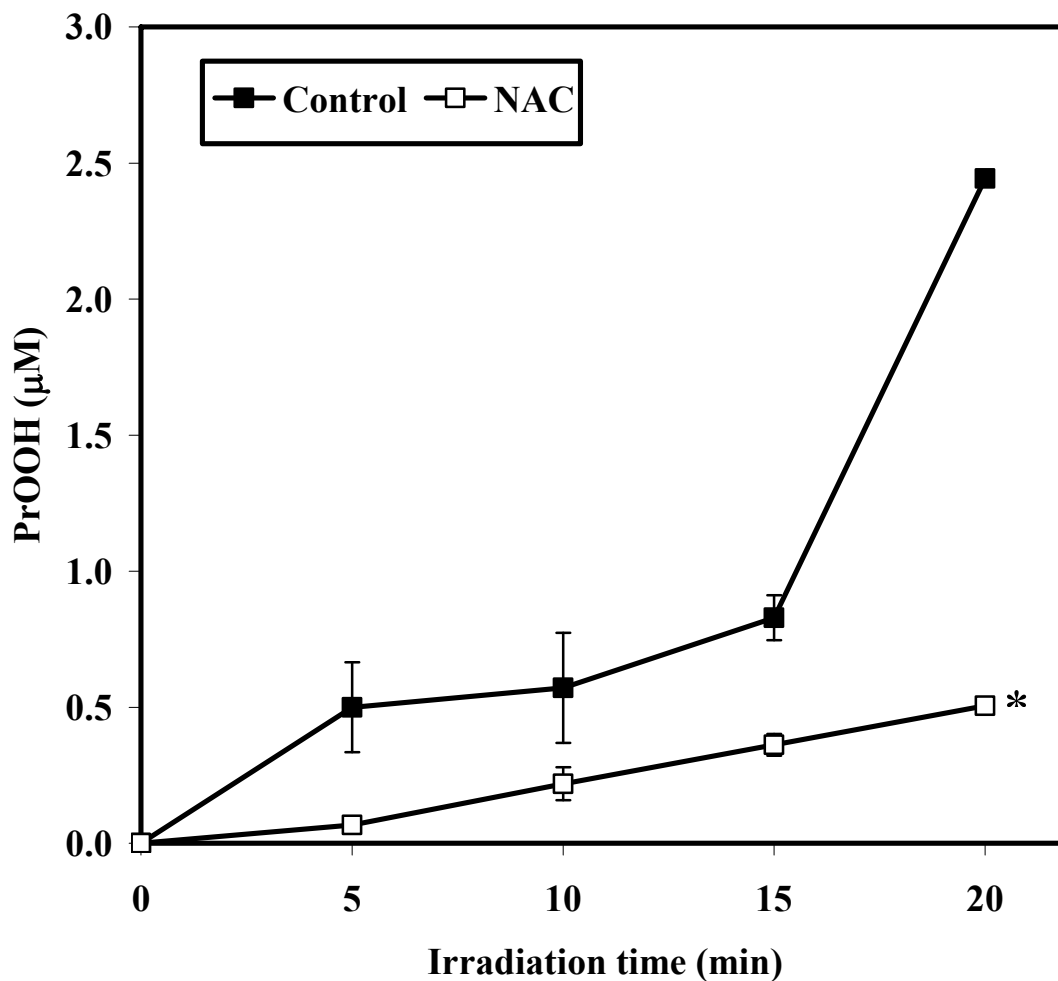
Figure 3.46 Effect of GSH enhancement on protein hydroperoxides formation in irradiated cells

The protein hydroperoxide concentrations were measured by G-PCA-FOX assay. Experimental details are in Section 3.9.5.4. The data points were derived from five experiments. * Indicates statistically significant difference between control and GSH-enhanced samples at $p<0.05$ level. Control: without NAC treatment; NAC: with NAC treatment.

3.9.6 Effect of 20 min irradiation on GSH levels after NAC and BSO treatment

3.9.6.1 Comparison of GSH depletion and repletion on intracellular GSH levels in γ-irradiated cells

L-Buthionine sulfoximine (BSO) has been used to reduce intracellular GSH levels in cells (Guaiquil, Farber et al. 1997). Increase of the intracellular GSH levels has been achieved with N-acetylcysteine (NAC) (Anderson 1997). HL-60 cells treated with 15 mM NAC or 100 μM BSO, and controls were irradiated for 20 minutes by gamma-irradiation. GSH levels were measured by Ellman's assay, and total protein analysis was performed by Bradford's assay. The comparison of GSH depletion and repletion on intracellular GSH levels in γ irradiated cells is shown in Figure 3.47. The level of GSH diminished after 20-min irradiation (45 Gy/min) in all three groups. The amounts of GSH reduction in average was 13.6 nmole GSH per mg of cell protein.
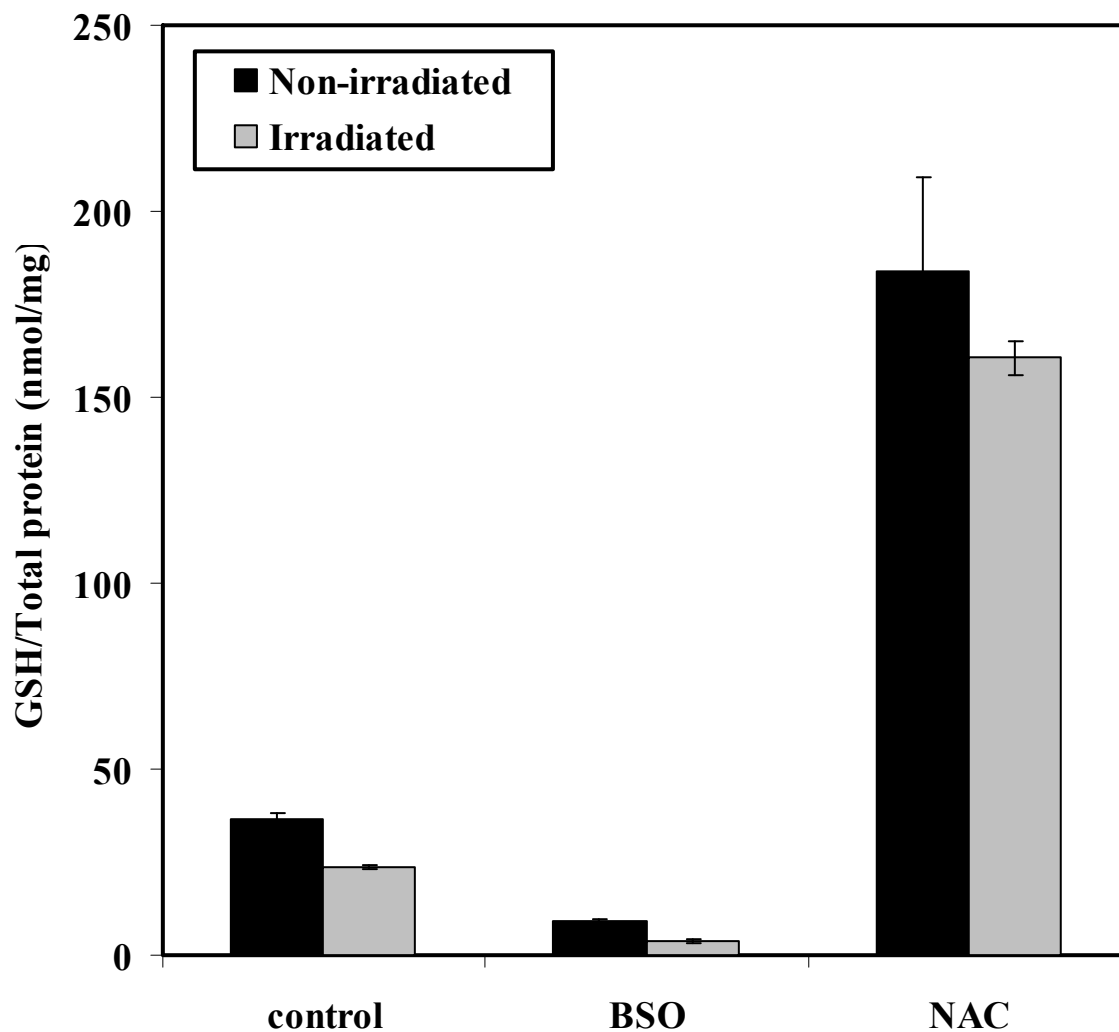
Figure 3.47 Comparison of GSH depletion and repletion on intracellular GSH levels in γ-irradiated cells

GSH Level was measured by Ellman's assay, and total protein analysis was performed by Bradford's assay. Experimental details are in Section 3.9.6.1. Each bar represents the mean ± SD of three experiments.

3.9.6.2 *Effect of GSH depletion and repletion on protein peroxide formation in HL-60 cells irradiated for 20 min*

HL-60 cells treated with 15 mM NAC or 100 μM BSO, and controls were irradiated at 0 and 20 minutes (45 Gy/min) by gamma-irradiation. After irradiation, the amount of protein peroxides in cells was measured by the G-PCA-FOX assay.

After 20-min irradiation, the amount of protein peroxides was significantly higher in BSO treated cells and was significantly lower in NAC treated cells compared with control cells (Fig. 3.48). These results showed that the GSH could significantly reduce the protein peroxide formation in HL-60 cells. GSH might be a potential antioxidant for protein peroxide damage in cells.
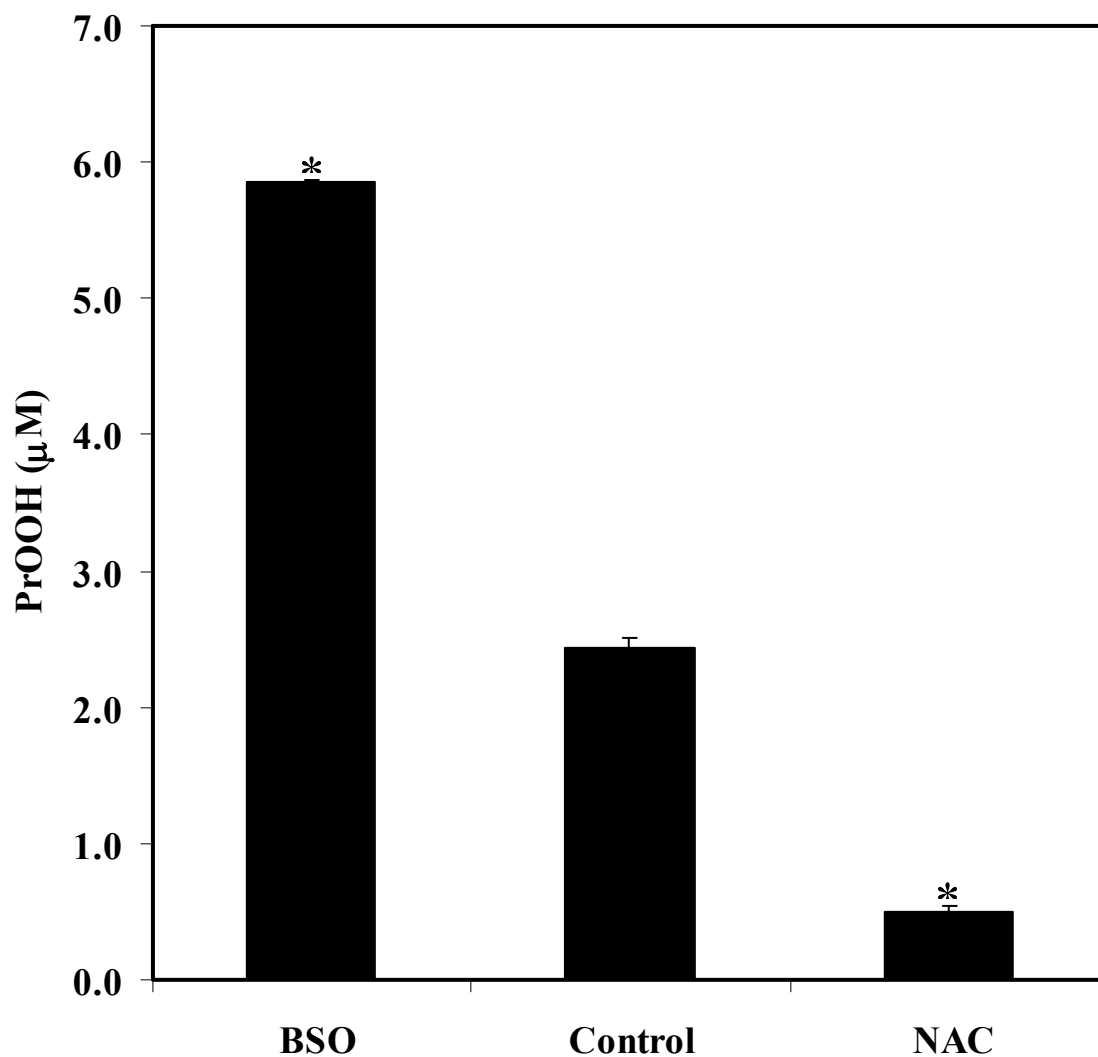
Figure 3.48 Effect of GSH depletion and repletion on protein peroxide formation in HL-60 cells

The amount of protein peroxides of cells was measured by the G-PCA-FOX assay. The data points were derived from five experiments and present the amount of protein peroxide formed. Experimental details are in Section 3.9.6.2. *: indicates statistically significant difference between control and irradiated samples at $p<0.05$ level. Control: without any treatment; NAC: with 15 mM NAC treatment, BSO: with 100 µM BSO treatment.

3.10 The role of ascorbate on protein hydroperoxide formation induced by gamma irradiation in HL-60 cells

3.10.1 Effect of ascorbate on cell viability during irradiation

HL-60 cells growing in exponential phase were harvested from serum-containing RPMI-1640 medium and suspended in the serum-free RPMI-1640 medium. For ascorbate treatment, HL-60 cells (2.5×10^6 cells/ml) in both media were treated with various concentrations of ascorbate (0.1-0.3 mM) in the presence of ascorbate oxidase (5 units) (which converted the ascorbic acid into dehydroascorbic acid (DHA) in less than 5 min) at 37 $^{\circ}$C for 30 minutes. Cell viability was greater than 95 % as determined by trypan blue exclusion. After incubation, control and treated cells were suspended in the medium again. Then the cells were irradiated at room temperature for 20 minutes at dose rate of 45 Gy/min. The viability of cells was measured by both trypan blue exclusion assay and MTT assay.

The viability data for control and irradiated HL-60 cells treated with various concentration of ascorbate is shown in Figure 3.49. In trypan blue exclusion assay, the cell viability was maintained at about 95 % in both control and irradiated cells with different amounts of ascorbate loading. In MTT assay, the cell viability was slightly reduced by the irradiation and not influenced by the ascorbate loading (Fig. 3.50). Both results suggest that the amounts of added ascorbate would not affect the cell viability in RPMI-1640 medium.
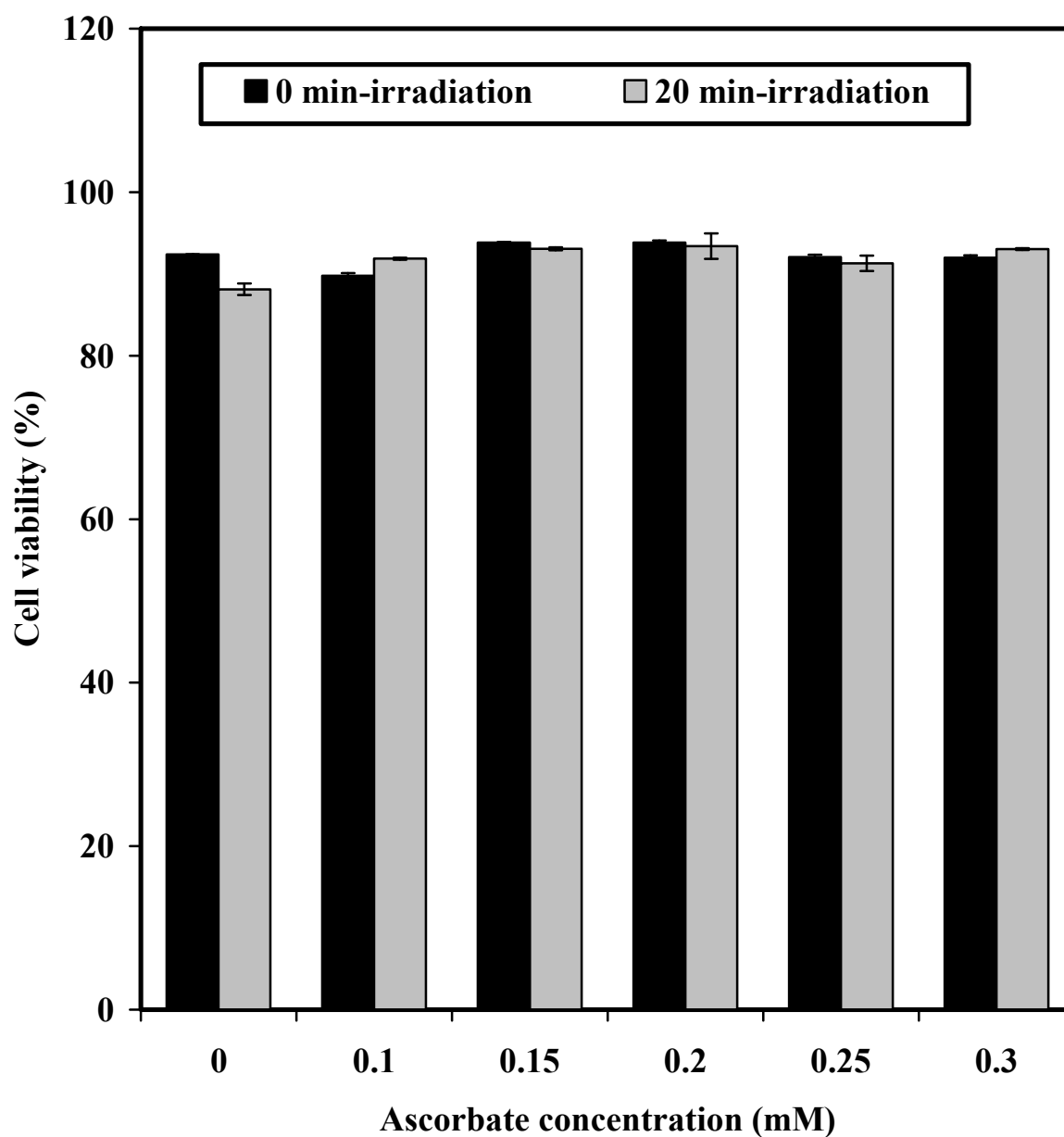
Figure 3.49 Effect of extracellular ascorbate on cells in RPMI-1640 medium using trypan blue exclusion assay

The viability of cells was measured by trypan blue exclusion assay. Experimental details are in Section 3.10.1. The data points were derived from three experiments.
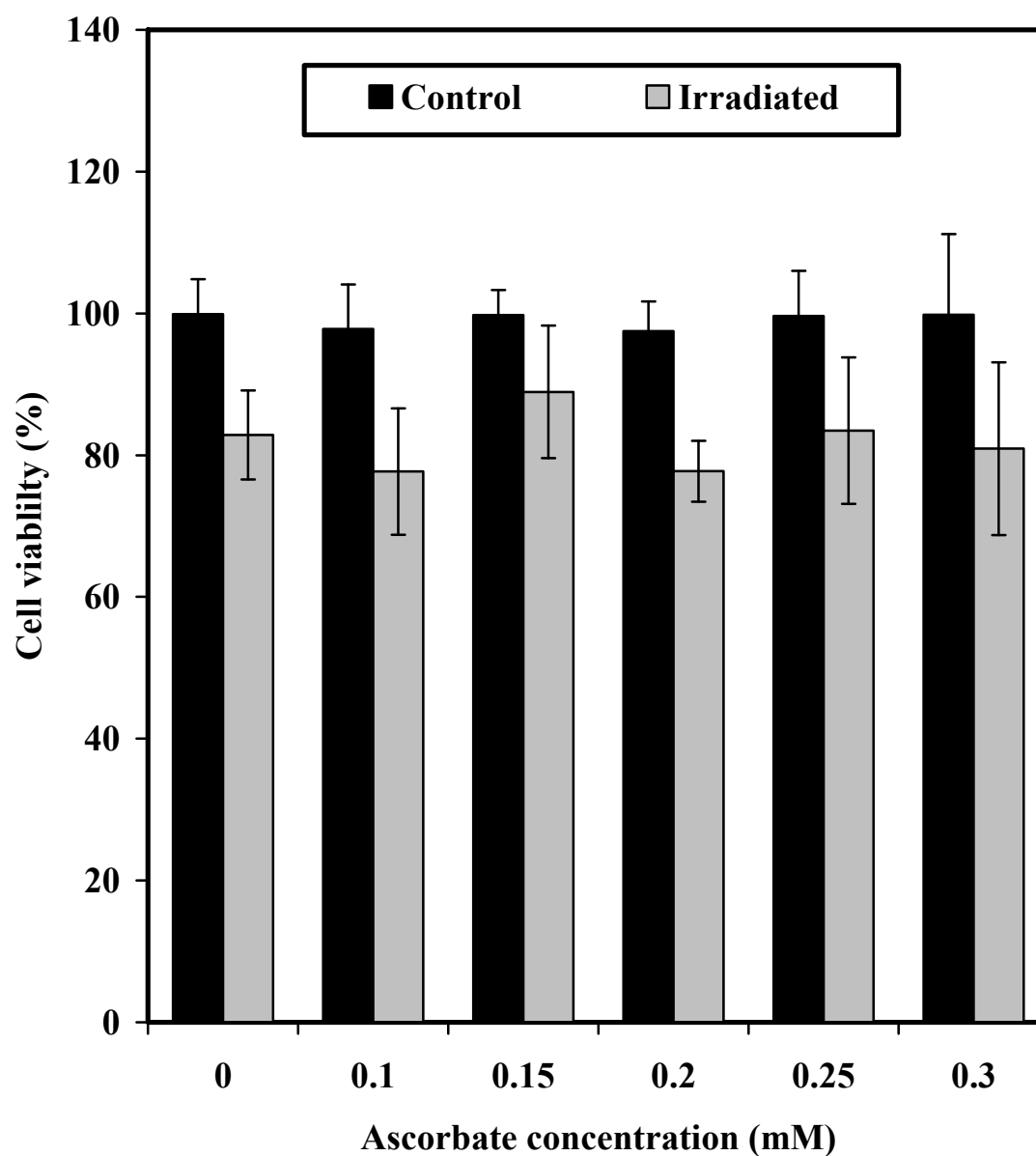
Figure 3.50 Effect of extracellular ascorbate on cells in RPMI-1640 using MTT assay

The viability of cells was measured by MTT assay. Experimental details are in Section 3.10.1. The data points were derived from three experiments.

3.10.2 Analysis of intracellular ascorbate in HL-60 cells

High-performance liquid chromatography was used to measure the amount of intracellular accumulation of ascorbic acid. This ascorbic acid technique provides the highest sensitivity and eliminates interference by substances (Levine, Wang et al. 1999). The detail of HPLC method used in these experiments is descry

ibed in Material and Methods section.

3.10.2.1 Ascorbic acid chromatography and standard curve

A standard curve displaying absorbance peak area as a function of a wide range of ascorbic acid concentrations is presented in Figure 3.51. It was generated over range from 0.25 nmol to 0.1 μmol of ascorbic acid per injection. The standard curve is linear, with correlation coefficient of 0.999.Ascorbaic acid amounts were expended as mAbs and correlated to concentration with the standard curve (Fig. 3.51). The base line remained stable. The ascorbic acid standards were prepared in 60 % methanol/1 mM EDTA, which stabilized the ascorbic acid without interfering with the chromatography.

Representative chromatography of 1 -10 nmole ascorbate (0.1 mM ascorbate was injected at 10-100 μl in HPLC) in solutions of 60 % methanol/water (v/v)/1 mM EDTA is shown in Figure 3.52. Ascorbate peak heights were dependent on the injection volume. The retention time for ascorbate was about 4.7 min and for EDTA was 6.4 min; therefore, the complete analysis time was as short as 10 min.
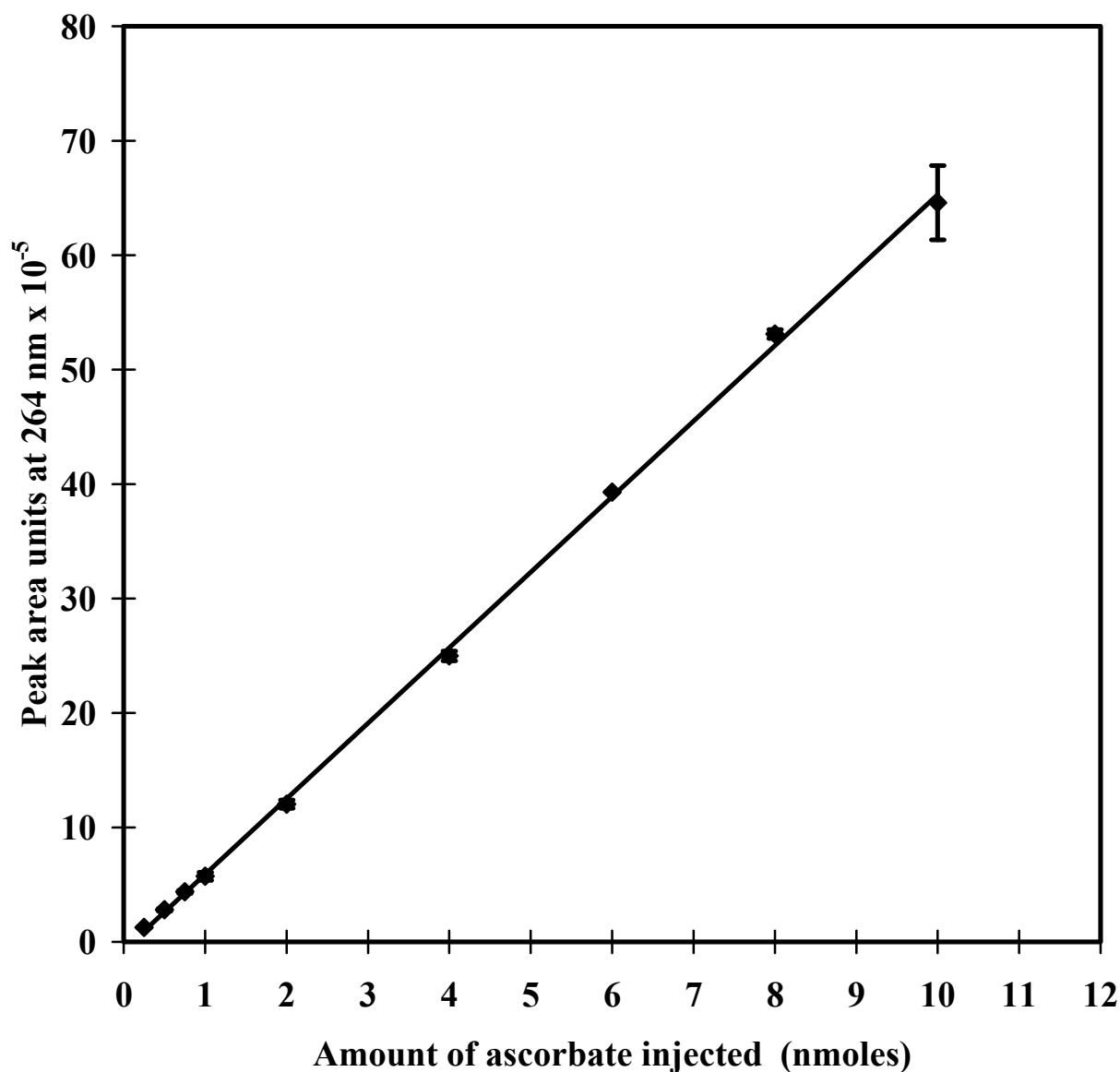
Figure 3.51 The standard curve of ascorbate in HPLC

Plot of area units vs moles ascorbate applied. Standards were prepared by dissolving ascorbate in 60/40 methanol/water (v/v) containing 1mM EDTA. Experimental details are in Methods and Section 3.10.2.1. The coefficient of the curve is y = 6.5984x - 0.6905, R^2 = 0.9994. Each data point represents the mean ± SD of three values.
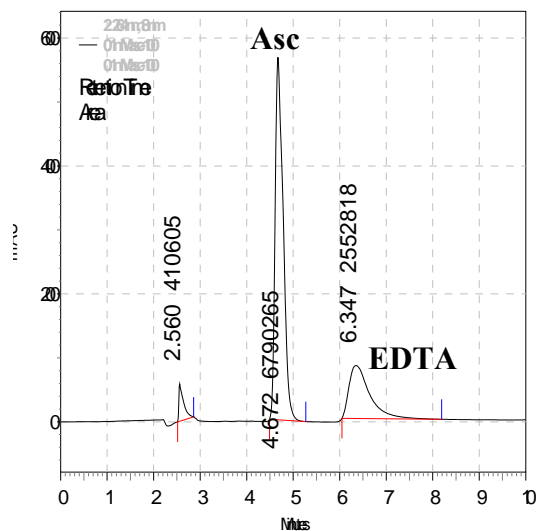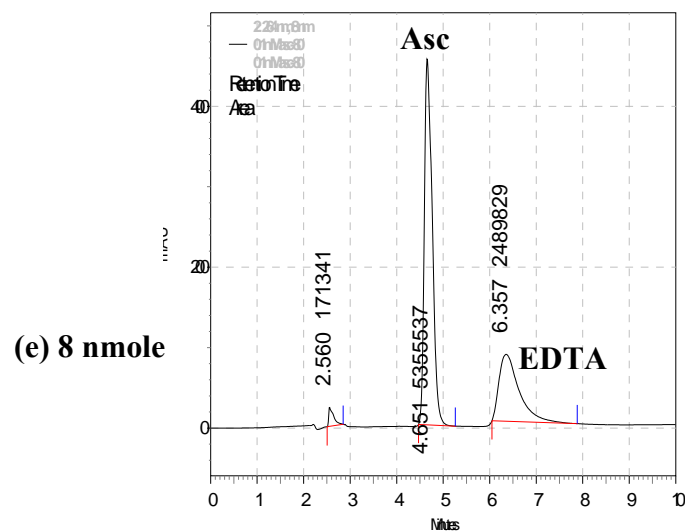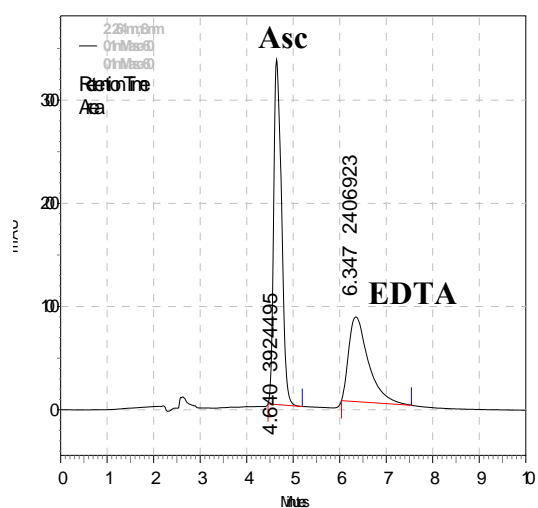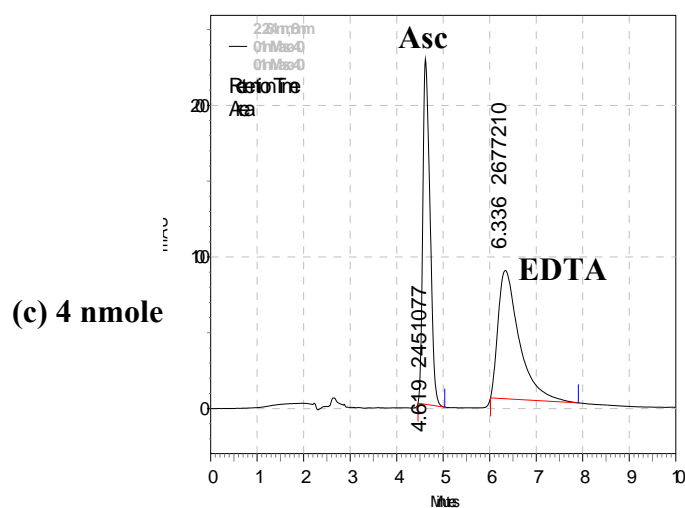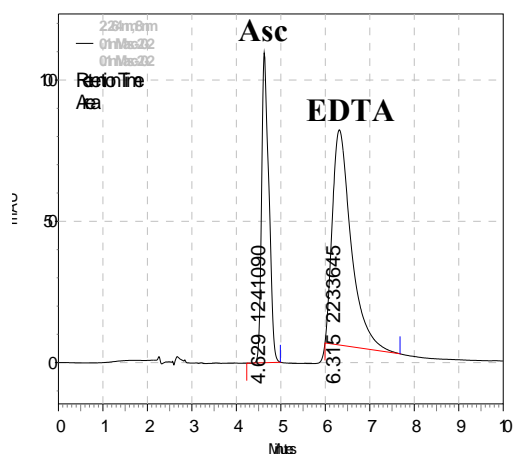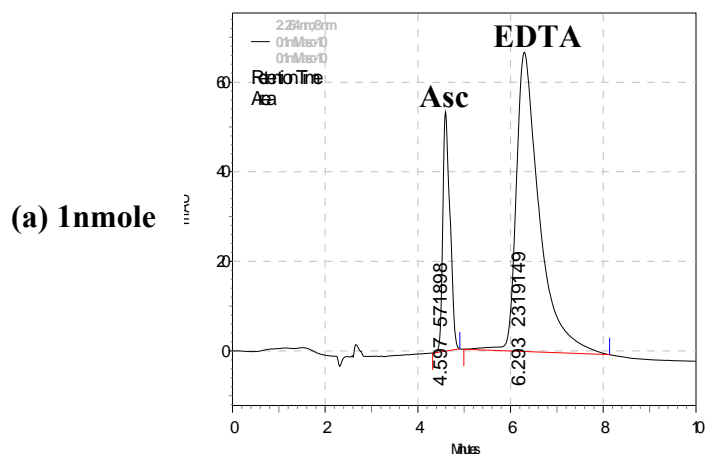
Figure 3.52 HPLC elution profiles of ascorbate

3.10.2.2 Intracellular accumulation of ascorbate in HL-60 cells

It has been reported that HL-60 cell line transports the oxidized form of ascorbate, DHA, and accumulates reduced ascorbic acid. Therefore, in cells treated with DHA, no extracellular ascorbate would be detected, only the intracellular form (Witenberg, Kalir et al. 1999). HL-60 cells, treated with different concentrations of ascorbic acid and 5 units of ascorbate oxidase, showed intracellular accumulation of ascorbate which was analyzed with the HPLC system.

HL-60 cells (2.5×10^6 cells/ml) in RPMI-1640 medium were treated with various concentrations of ascorbic acid (0.1-0.3 mM) in the presence of ascorbate oxidase (5 units). The cells were then irradiated at room temperature for 0 and 20 min at dose rate of 45 Gy/min. The intracellular accumulation of ascorbic acid was analyzed by HPLC. The analyzed volume was 100 µl.

An estimated intracellular volume of 0.3 µl per 10^6 HL-60 cells was used to express the concentration of ascorbic acid in the cells (Welch, Wnaf et al. 1995). The cellular accumulation of ascorbic acid at different irradiation times are shown in Figure 3.53 and summarized in Table 3.23. In the presence of 0.3 mM ascorbate, the HL-60 cells contained 28.0 mM ascorbic acid. After irradiation, the intracellular concentration of ascorbate was significantly reduced. The intracellular concentration of ascorbate was 1.3-2.6 folds lower in irradiated cells compared with control cells. Figure 3.54 shows that the ascorbic acid peak area of control cells was 1.4 fold greater than irradiated cells in the chromatography

results. Therefore, the intracellular ascorbate levels were significantly lowered by irradiation, demonstrating its consumption by the radiation-generated radicals.

Table 3.23 Intracellular ascorbic acid in HL-60 cells analyzed by HPLC analysis

Extracellular Asc concentration (mM)	Intracellular ascorbic acid (mM)	
	Control	Irradiated
0.10	4.1	1.7
0.15	6.2	2.4
0.20	13.2	6.8
0.25	21.8	11.2
0.30	28.0	18.7

* Control: cell without irradiation; irradiated: cells after 20-min irradiation.
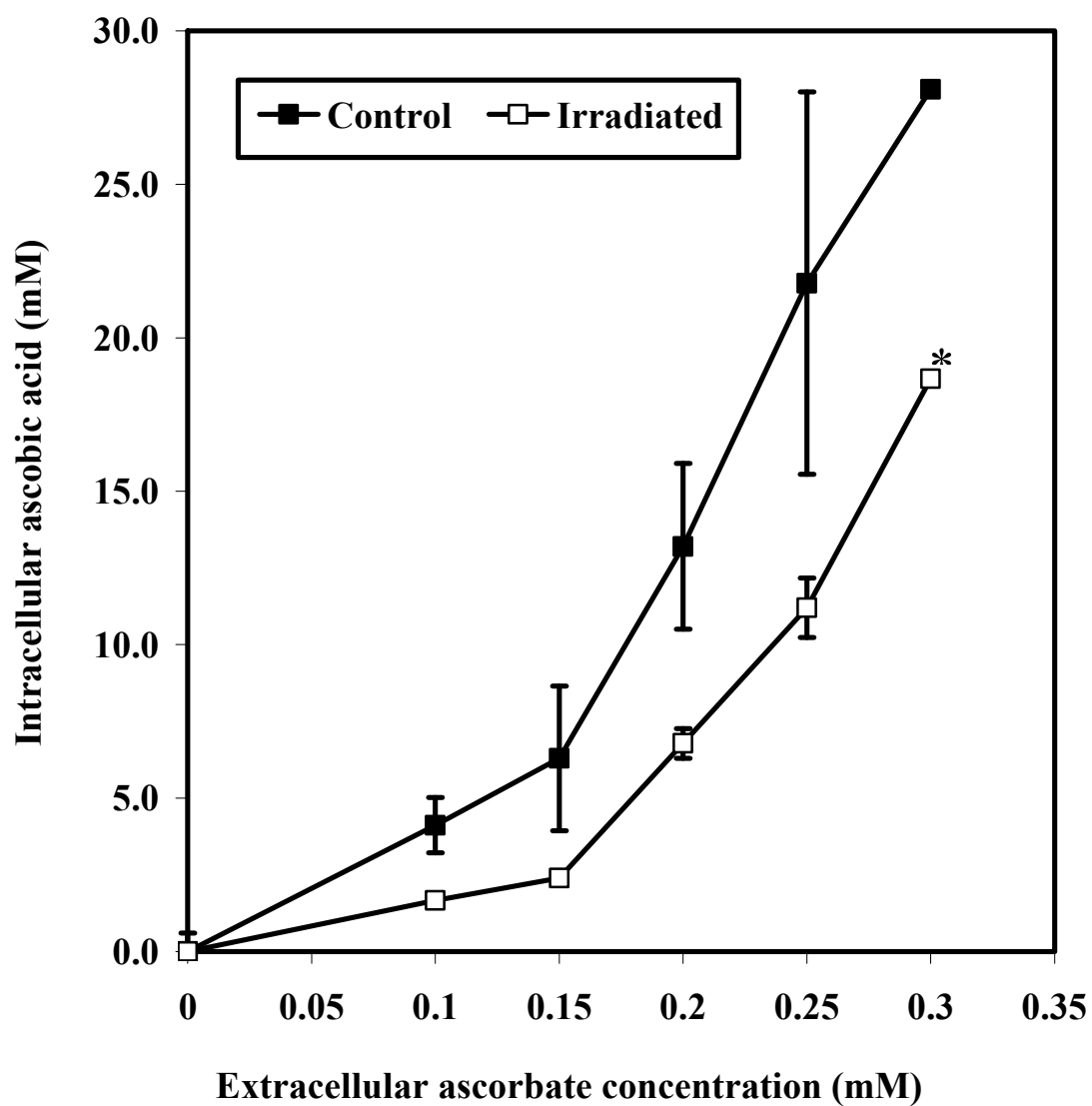
Figure 3.53 Intracellular accumulation of ascorbate in RPMI-1640 medium

The intracellular accumulation of ascorbic acid was analyzed by HPLC. Experimental details are in Section 3.10.2.2. The data points were derived from five experiments. *: $p<0.05$.
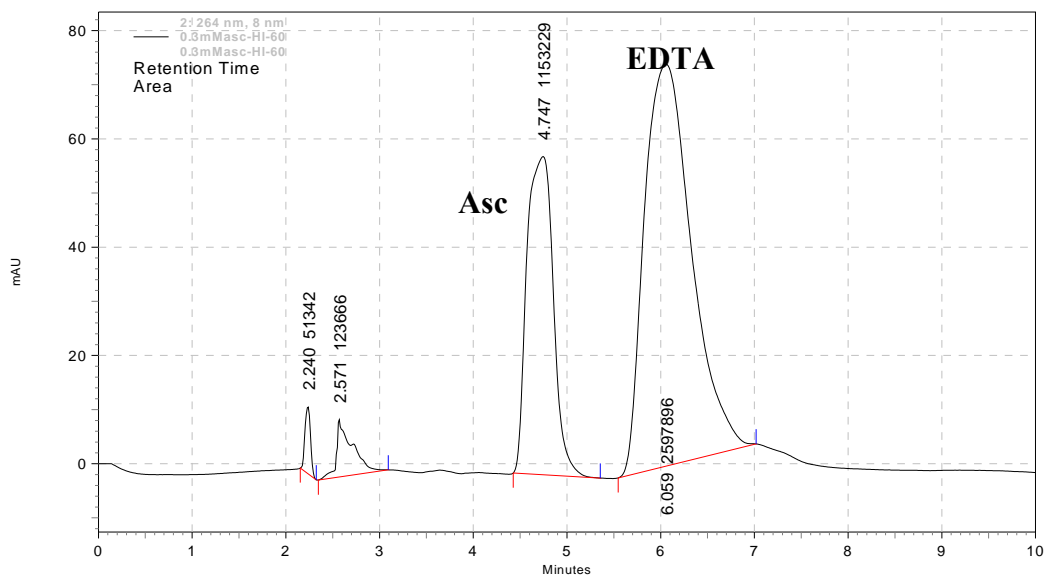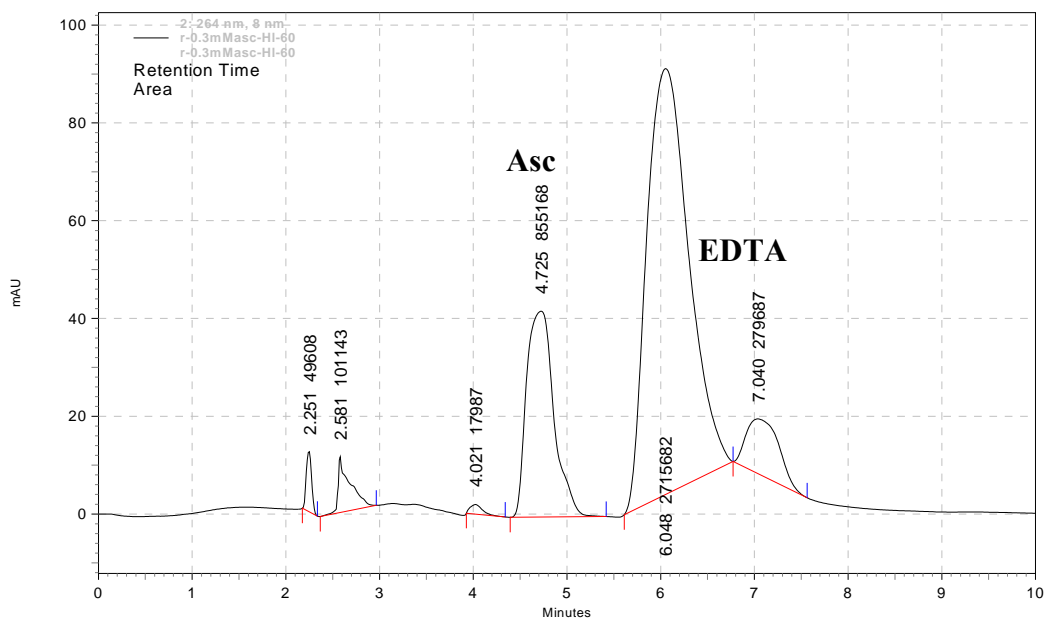
Figure 3.54 HPLC elution profile of ascorbic acid in HL-60 cells incubated with RPMI-1640 medium

The intracellular accumulation of ascorbic acid was analyzed by HPLC. The analyzed volume was 100µl. Experimental details are in Section 3.10.2.2.

3.10.3 The effect of ascorbate on protein hydroperoxide formation

HL-60 cells (2.5×10^6 cells/ml) in RPMI-1640 medium were treated with various concentrations of ascorbic acid (0.1-0.3 mM) in the presence of ascorbate oxidase (5 units). The cells were then irradiated at room temperature for 0 and 20 min at dose rate of 45 Gy/min. After irradiation, the cells were washed with PBS before G-PCA-FOX assay for protein hydroperoxides.

With RPMI-1640 incubation, ascorbate reduced the cellular protein hydroperoxide production in a dose-dependent matter (Fig. 3.55). The hydroperoxide concentrations in γ-irradiated cells were determined by the FOX assay the results were given in Table 3.24. For the 0.3 mM extracellular ascorbate-loaded cells after 20-min irradiation, the hydroperoxide levels were reduced statistically significantly using two-tailed t-test ($p<0.05$). The cellular protein hydroperoxide formation is plotted against various concentrations of extracellular ascorbate in Figure 3.56.

Table 3.24 Hydroperoxide yields in 20 min-irradiated cells in the presence or absence of ascorbate

Systems	PrOOH (μM)
Without ascorbate	2.50 ± 0.44
With 0.3 mM extracellular ascorbate	$*0.73 \pm 0.23$

*: indicates statistically significant difference to the cells without ascorbate treatment at $p<0.05$ level. PrOOH: protein peroxides.
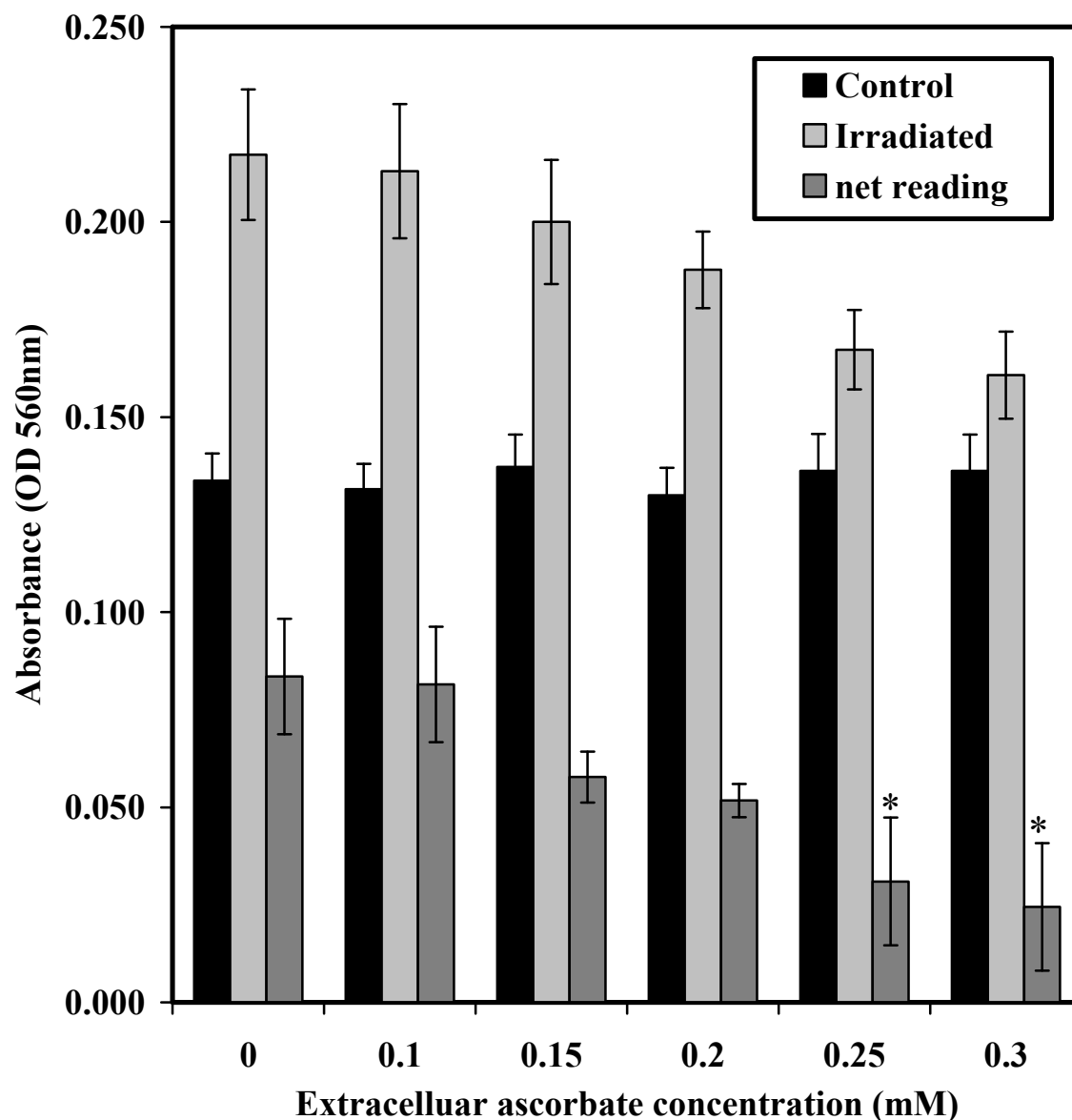
Figure 3.55 Effect of ascorbic acid on the formation of protein hydroperoxides in RPMI-1640 medium

Protein peroxide concentrations of cells were measured by G-PCA-FOX assay. Experimental details are in Section 3.10.3. The data points were derived from five experiments. *: $p < 0.05$. Net reading is the absorbance difference between control and irradiated cells.
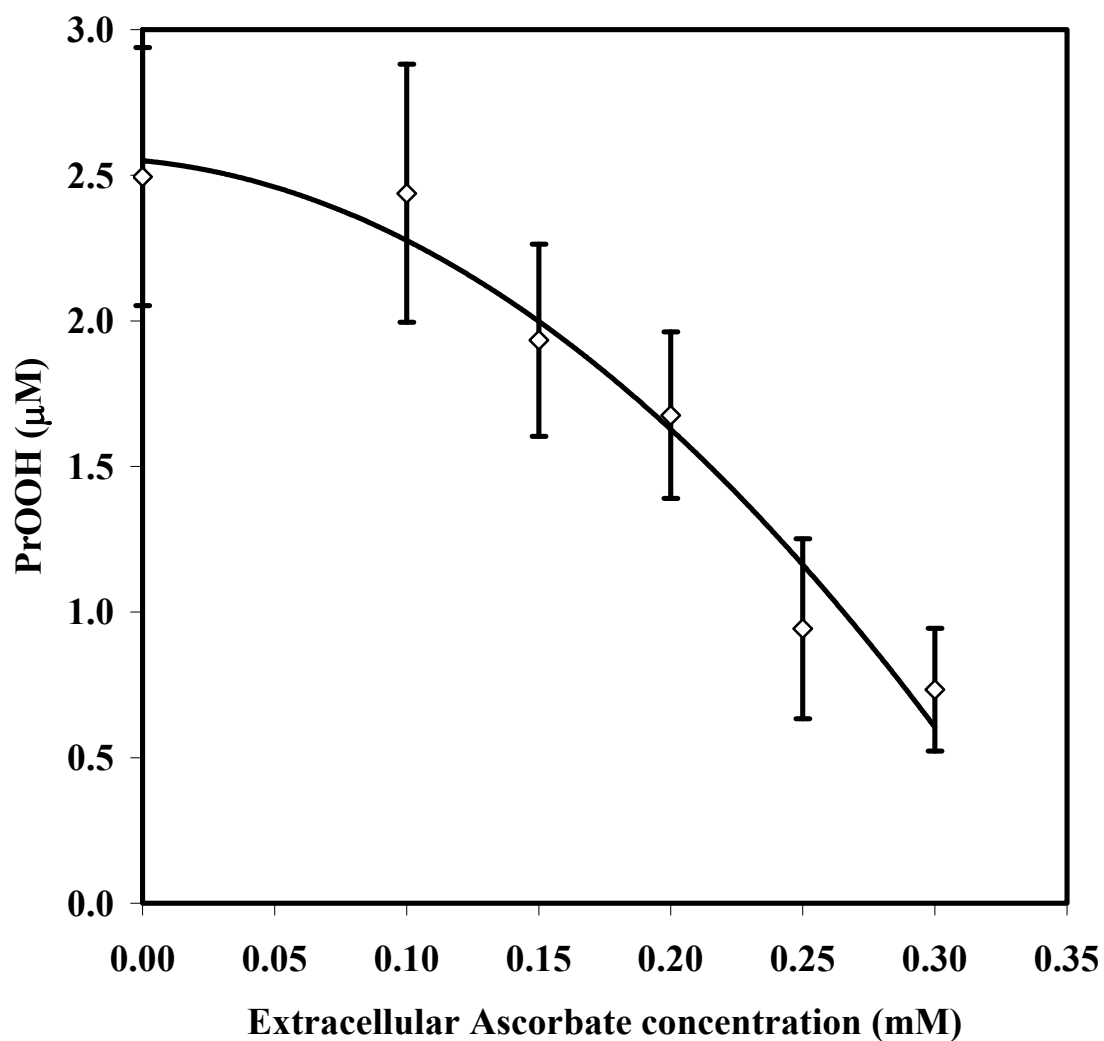
Figure 3.56 The cellular protein peroxide formation in RPMI-1640 medium

Protein hydroperoxide concentrations in cells were measured by G-PCA-FOX assay. Experimental details are in Section 3.10.3. The results plotted show the relationship between peroxide levels and extracellular ascorbate. The data points were derived from five experiments.

CHAPTER 4

DISCUSIONS

SECTION (I) IN VITRO STUDIES

4.1 Different radicals produced under different gases

^{60}Co γ-irradiation induced the radiolysis of water (reaction 3.15) and produced e_{aq}^-, $HO^\bullet$, and $H^\bullet$. The yield of $H^\bullet$ is less than 10 % of the yield of $HO^\bullet$ and e_{aq}^- (Table 1.3); therefore, $HO^\bullet$ and e_{aq}^- are more important species in this process. Under different experimental gas saturations, different radicals are formed (Davies 1987):

1. Under 100 % N_2O, the solvated electron (e_{aq}^-) reacts quantitatively to produced more $HO^\bullet$ (reaction 3.18).

2. In the presence of O_2, e_{aq}^- reacts to produce to $O_2^{\bullet-}$, and $H^\bullet$ reacts to form $HO_2^\bullet$. The $HO_2^\bullet$ produced is rapidly deprotonated at neutral pH to form more $O_2^{\bullet-}$ (reaction 3.16 and 3.17).

3. Under O_2 with sodium formate, $^\bullet HO$ reacts to produce $O_2^{\bullet-}$ only (reaction 3.13 and 3.14).

The use of ^{60}Co γ-irradiation provides the best control of the nature and quantities for free radical generation. Under my condition, all energy absorbed in the irradiated solutions led to decomposition of water and, for 100 eV absorbed, to the formation of 2.8 $HO^\bullet$ and 3.2 $O_2^{\bullet-}$ in the presence of oxygen, under anaerobic conditions only $HO^\bullet$ is formed (Gebicki and Gebicki 1993). As the yields of these primary radicals are known form the radiation, dose rate and irradiation time, this system makes it possible to obtain a material balance

under "substrate-saturating " conditions, in which all primary radicals react with substrate rather than with each other (Neuzil, Gebicki et al. 1993). In my case, ascorbate or protein acts as the substrate. The modification and oxidation of these molecules were studied.

4.2 Ascorbate oxidation by γ-irradiation generated free radicals

4.2.1 Ascorbic acid free radicals

Ascorbic acid is a di-acid, which has two ionizable-OH groups (Fig. 4.1). Since pK_{a1} is 4.1 and pK_{a2} is 11.8, 99.5 % of ascorbic acid is present as ascorbate (AscH$^-$) at the physiological pH 7.4. In acid solution, ascorbic acid will be present as ascorbic acid (AscH$_2$) (Halliwell and Gutteridge 1999).
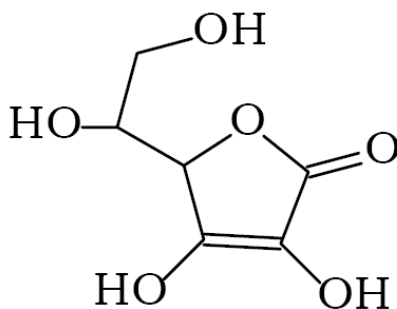
Figure 4.1 Ascorbic Acid Structure (AscH$_2$)

Donation of one electron, reversible, by ascorbate gives an oxidized radical, the resonance-stabilized tricarbonyl ascorbate free radical (Asc$^{\bullet-}$) (Fig. 4.2) (Carr and Frei 1999), which can be further oxidized to give dehydroascobate (DHA). Because Asc$^{\bullet-}$ has its unpaired electron in a highly delocalized π-system, it is a reactively unreactive free radical. The

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ascorbate free radical is a strong acid, having a pKa of -0.86. Therefore, it will exist as a monoanion over the entire biological pH range. In additiom, the Table 4.1 showed that ascorbate can not only neutralize hydroxyl (HO$^\bullet$), alkoxyl (RO$^\bullet$) and peroxyl (ROO$^\bullet$) radicals by hydrogen donation, but also also neutralize the radical form of other antioxidants, such as glutathione (GS$^\bullet$) and Vitamin E (tocopherol) (Toc$^\bullet$) ($E'^\circ = 1.55 \times 10^6$ M^{-1}s^{-1}) (Buettner 1993):

$$\text{AscH}^- + \text{Toc}^\bullet \rightarrow \text{Toc} + \text{Asc}^{\bullet-} \qquad\qquad (4.1)$$

These properties make ascorbate an outstanding donor antioxidant (Buettner and Jurkiewicz 1996).
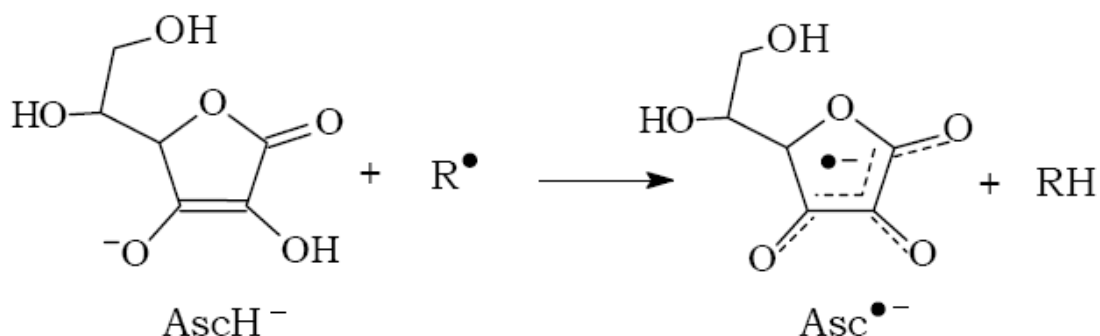
Figure 4.2 Ascorbate is a donor antioxidant (where R$^\bullet$ is any of these oxidizing free radicals)

Table 4.1 Some rate constants of ascorbate reaction with different oxygen radicals

Chemical species	Reaction rate $(M^{-1}s^{-1})$ (pH 7.4)
Hydroxyl radical, $HO^\bullet$	1.1×10^{10}
Alkoxyl radical, $RO^\bullet$	1.6×10^9
Peroxyl radical, $ROO^\bullet$	$1\text{-}2 \times 10^6$
Superoxide anion/ hydroperoxyl radical, $O_2^{\bullet-}/HO_2^\bullet$	2.7×10^5
Glutathiyl radical, $GS^\bullet$	6×10^8 (pH 5.6)
$Asc^{\bullet-}$ (dismutation)	2×10^5
Tocopheroxyl radical, $TO^\bullet$	2×10^5

(Modified from Carr, A. and Frei, B. 1999)

Dehydroascobate is the product of two-electron oxidation of ascorbate via dismutation of ascorbate radical (Reaction 4.2). This dismutation reaction is the principal route to the elimination of the $Asc^{\bullet-}$ *in vitro*. However, *in vivo* it is thought that reducing enzymes are involved in the removal of this radical, resulting in the recycling of ascorbate (Hossain and Asada 1985). Moreover, $AscH^-$ is readily regenerated from $Asc^{\bullet-}$ with NADH or NADPH-dependent reductases (Hossain and Asada 1985).

$$2\ Asc^{\bullet-} + H^+ \rightleftarrows AscH^- + DHA \qquad\qquad (4.2)$$
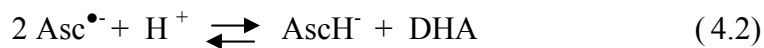
The ascorbate free radicals have been detected by electron paramagnetic resonance spectroscopy (EPR) at low steady-state levels in biological and tissue samples, such as plasma (Sharma and Buettner 1993), synovial fluid (Buettner and Chamulitrat 1990), and skin (Jurkiewicz and Buettner 1994). In most biological experiments the actual steady-state concentrations of $Asc^{\bullet-}$ are $\sim10^{-7}$ M, often 10^{-7}-10^{-10} M (Buettner 1990). As oxidative stress increases in a system, the steady-state $Asc^{\bullet-}$ concentration increases. The intensity of EPR spectrum of $Asc^{\bullet-}$ can be used as an noninvasive indicator of oxidative stress *in vitro* and *in vivo* (Buettner and Jurkiewicz 1996).

4.2.2 Quantitation of ascorbic acid/ascorbate

Ascorbic acid solutions are colourless as neither diacid nor the monoanion has significant absorbance in the visible region of the spectrums, allowing simple and convenient UV spectrophometric quantification.

1. Ascorbate: The peak of the absorption curve for ascorbate is 265 nm. The molar absorption coefficient is 14,500 $M^{-1}cm^{-1}$ in near neutral buffered aqueous solutions (Buettner 1988).

2. Ascorbic acid: The diacid has an absorption spectrum with approximately $\varepsilon_{244} = 10,800$ $M^{-1}cm^{-1}$ in acid aqueous solution (Buettner and Jurkiewicz 1997).

In biochemical and biological samples, an immense number of substances absorb in the same wavelength region, often making direct UV detection unfeasible. However, in the simple chemical test tube systems, direct detection can be used to great advantage (Buettner and Jurkiewicz 1997).

For my experiments, ascorbate concentrations could be measured by the absorbance at 265 nm, while ascorbic acid concentrations are measured at 244 nm. A wide range of molar absorption coefficients have been reported, ranging from 7,500 to 20,400 $M^{-1}cm^{-1}$ (Buettner and Jurkiewicz 1997). I find that ε_{265} = 14,500 $M^{-1}cm^{-1}$ (Fig. 3.5) best reflected my experimental observations when doing the experiments in pH 7.0 buffered aqueous solution. The result is slightly lower than the molar coefficient given by Buettner in 1990.

In my experiments, ascorbate was dissolved in neutral phosphate buffered solutions; however, the measurement of ascorbate concentrations was performed by the addition of PCA to 2 M concentration, which acidified the solutions and turned ascorbate to ascorbic acid. Thus, the molar coefficient at 244.5 nm was measured at 8,230 $M^{-1}cm^{-1}$ (Fig. 3.6) in the acid solution in my experiments. This result was reproduced in several experiments.

To sum up, concentration of ascorbate can be determined by the absorbance of its absorbance at 265 nm in neutral pH and at 244.5 nm in acid solution in good agreement with previously published results.

4.2.3 Gamma irradiation-induced oxidation of ascorbate

Monitoring the decrease in the 265 nm or 244.5 nm absorbance of ascorbate and ascorbic acid is a quick and easy method that uses readily available equipment. As seen in Figure 3.7, the maximum amount of ascorbate oxidized by gamma-irradiation in aqueous solution after 79.4 Gy dose was 19.4 μM. Measurements with higher amount of ascorbate (30 to 50 μM) in sample solutions resulted in the level of oxidized ascorbate remaining the same.

The ascorbate was oxidized in reaction 4.3 and 4.4 (Bielski, Comstock et al. 1971; Nishikimi 1975).

$$AscH^- + HO^\bullet \rightarrow H_2O + Asc^{\bullet-} \qquad\qquad (4.3)$$

$$AscH^- + O_2^{\bullet-} \rightarrow H_2O_2 + Asc^{\bullet-} \qquad\qquad (4.4)$$

The G value of oxidized ascorbate by combined $O_2^{\bullet-}$ and $HO^\bullet$ is 6.0. In 1970, Bielski and Allan reported that ascorbate has been found to be oxidized very rapidly by $HO^\bullet$ radicals formed in aqueous solution by radiolysis. They also assumed that every radical formed by irradiation of water, whether e_{aq}^- and $HO^\bullet$ would results in formation of one oxidized ascorbate radical. The total yield of radicals in water is $G = 6.05$ and is about 0.4 higher in nitrous oxide saturated solution. Therefore, it was assumed that the yield of ascorbate radicals was $G = 6.45$ (Bielski and Allen 1970). In Bielski's report, he measured the amount of ascorbate radicals and also made some assumptions to predict the yield of ascorbate free radicals. In my experiments, only the yield of oxidized ascorbate was measured which corresponds with ascorbate free radical and DHA formation.

The weak (UV-Vis) absorption spectrum of ascorbate radical (at pH 6.4, λmax $=360$nm, $\varepsilon_{360} = 3,300$ $M^{-1}cm^{-1}$) makes it impossible to observe it directly in steady-state experiments. Consequently, EPR spectroscopy of $Asc^{\bullet-}$ is the preferred method for observing it (Bors and Buettner 1997). However, the aims of my experiments were to study the effect of protein radicals on ascorbate. The studies of ascorbate radicals themselves were less important.

4.2.4 Gamma irradiation-induced oxidation of ascorbate under different gases

As shown in Table 3.2, the G value of ascorbate oxidation was 3.3 under air; 2.5 under N_2O, and 1.8 under argon after 60-second irradiation. Under argon (only $HO^\bullet$), 67 % of radicals oxidized ascorbate, rest was lost in side reactions. Under air, 56 % of $O_2^{\bullet-}$ and $HO^\bullet$ oxidized ascorbate and remaining radicals were lost in side reactions. Under N_2O, only 45 % of $HO^\bullet$ oxidized ascorbate. The side chain reactions includ $HO^\bullet$-$Asc^{\bullet-}$ and $HO^\bullet$-$HO^\bullet$ radical-radical reactions. The rate of formation of the ascorbate radicals by $HO^\bullet$ is very fast and produces large amount of ascorbate radicals. These ascorbate radicals in the presence of phosphate buffer decay, and then suggest a mechanism in which the ascorbate radical ion is in equilibrium with a dimer. This dimer reacts with hydrogen ion, or with other proton donors within water and buffers, to form the disproportionation products ascorbate ion and dehydroascorbate acid (Bielski, Allen et al. 1981). This consequence might be the reason why in doubling the yield of $HO^\bullet$ under N_2O, the level of ascorbate oxidation was not doubled.

In the presence of oxygen, the amount of oxidized ascorbate was two fold higher than under argon. Ascorbate is oxidized by $O_2^{\bullet-}$ or its conjugated acid $HO_2^\bullet$ with rate constant is $2.7 \times 10^5 \ M^{-1}s^{-1}$ (Table 4.1) and produces ascorbate radicals (reaction 4.4). The ascorbate radicals can further react with $O_2^{\bullet-}$ and generate dehydroascrobate (DHA).

$$O_2^{\bullet-} + Asc^{\bullet-} \rightarrow H_2O_2 + DHA \qquad\qquad (4.5)$$

Yamazaki and his colleagues in 1968 reported that ascorbate radical is unable to reduce O_2 to $O_2^{\bullet-}$ (Yamazaki, et al. 1986). Therefore, the reaction 4.5 was assumed to proceed faster than reaction 4.4 under pH 7.4, with the rate of $O_2^{\bullet-}$ disappearance two times the rate of reaction 4.4 (Nishikimi 1975). Besides, the rate constant for ascorbate radical reactions with $O_2^{\bullet-}$ is 2.6×10^8 $M^{-1}s^{-1}$ which is 1.9×10^2 fold greater than with $AscH^-$ (1.4×10^8 $M^{-1}s^{-1}$) itself (Bors and Buettner 1997). Thus, $O_2^{\bullet-}$ radicals were more effective in oxidizing ascorbate than the excess of $HO^{\bullet}$ in my systems.

4.3 Ascorbate oxidation by protein radicals

The chemical changes that irradiation causes in proteins are, fragmentation, cross-linking, aggregation, and oxidation by ROS generated in the radiolysis of water (Garrison 1987; Davies and Dean 1997). Generally, exposure of proteins to ROS results in non-random and random fragmentation (Kempner 1993). The protein fragmentation in aqueous solution is determined by the local conformation of a particular amino acid in the protein, its accessibility to water radiolysis, and primary amino acid sequence (Filali-Mouhim, Audette et al. 1997). In a previous study, it has been reported that BSA was cleaved by the oxidative destruction of proline residues, yielding specific protein fragments. Besides, there have been reports on the aggregation and cross-linking of BSA by irradiation (Schuessler and Schilling 1984; Puchala and Schuessler 1993; Gaber 2005).

The initial attack on proteins by the radicals generated by water radiolysis results in formations of protein radicals. The most common pathway for the oxidation of proteins

involves the hydroxyl radical-mediated abstraction of a hydrogen atom to form a carbon-centered radical at the alpha-position of the amino acid residue in the polypeptide chain, or addition to the aromatic amino acid rings. Addition of O_2 to the carbon-centered radicals leads to formation of peroxyl radical derivatives, which upon decomposition lead to production of NH_3 and alpha-ketoacids, or to production of NH_3, CO_2, and aldehydes or carboxylic acids (Garrison 1987). As the number of carbon atoms in the amino acid side chains is increased, hydrogen abstraction at other positions in the carbon chain becomes more important and leads either to the formation of hydroxyl derivatives, or to amino acid cross-linked products as a consequence of carbon-centred radical recombination processes (Stadtman 1993). These protein radicals can be detected by electron spin resonance (ESR) spectroscopy (Ostdal, Davies et al. 2002). Amino acid hydroperoxide formation was also been detected in several proteins exposed to hydroxyl or peroxyl radicals (Gebicki, 2006).

The biological significance and the role of such protein radical formation *in vivo* are unidentified at present. These radicals may have a relatively long half-life which suggests they can act as oxidants. In particular, their reaction with low-molecular weight antioxidants and amino acids may provide of their potential biological significance (Ostdal, Skibsted et al. 1997; Ostdal, Andersen et al. 1999; Nauser, Koppenol et al. 2005).

In my studies, the ability of proteins radical in ascorbate oxidation *in vitro* was investigated. In all experiments, the concentration of protein and ascorbate were adjusted to ensure that over 95 % of the hydroxyl radicals from water radiolysis reacted with the protein to

produce protein radicals, which oxidize ascorbate in solution. This was done by setting the ratio of reaction rate of hydroxyl radicals with proteins and ascorbate at over 100: 1.

4.3.1 Effect of different gases

4.3.1.1 BSA-derived protein radicals

Different secondary free radicals were formed under various atmospheres by scavenging diverse primary radicals. As shown in Table 3.5, calculation based on the rate constants of the reactions of $HO^\bullet$ and $O_2^{\bullet-}$ with ascorbate predict that under conditions of complete radical scavenging, the $HO^\bullet$ should be responsible for 98 % of the ascorbate oxidation (Buettner and Jurkiewicz 1997). The much lower value actually found suggests that a considerable proportion of the $HO^\bullet$ did not react with ascorbate but disappeared in other processes. This is suggested by the results in Figure 3.8 showing that 20 μM ascorbate scavenged only a proportion of the $HO^\bullet$ generated. The presence of 500 μM BSA results in complete scavenging of $HO^\bullet$ to generate protein radicals able to oxidize ascorbate (Table 3.5). This increased the 87.5 % of ascorbate oxidized by the $HO^\bullet$ and $O_2^{\bullet-}$ generated by 120-sec irradiation to 99.6 % by the mixture of $O_2^{\bullet-}$ and $BSA^\bullet$ radicals. $BSA^\bullet$ radicals oxidized 7.2 μM ascorbate, while $BSAOO^\bullet$ oxidized 12.7 μM ascorbate (Table 3.5). In the presence of dioxygen, the ascorbate was probably oxidized by the protein peroxyl, not carbon-centred radicals. This is an original observation in my studies. The exact mechanism would need to be tested by comparing results obtained at different levels of dioxygen.

In the previous studies, the main focus of gamma-irradiation of BSA solution was on conformational changes in the disruption of the ordered structure of protein molecules such as degradation, cross-linking, and aggregation of the polypeptide chains due to ROS generation by water radiolysis, altered secondary structure and molecular profile of the BSA (Davies, Delsignore et al. 1987; Gaber 2005). In 2002, Østdal and his colleagues reported the reactivity of BSA radicals towards different biomolecules. They found out that proteins radicals have to be considered as dynamic species during oxidative processes in biological systems and that protein radicals should not be considered as end-products, but rather as reactive intermediates during oxidative processes in biological systems (Ostdal, Davies et al. 2002).

In 1984, Schuessler and Schiling irradiated BSA under aerobic and anaerobic conditions and studied the results by SDS-polyacrylaminde gel electrophoresis. They found that under anaerobic condition (HO$^\bullet$ only), loss of BSA protein by irradiation was caused by aggregation, only partly caused by changes in the intermolecular S-S bonds. Radiolysis under aerobic condition (HO$^\bullet$, O$_2$$^{\bullet-}$ and O$_2$) led to BSA loss by cleavage of the peptide chains, yielding protein fragments. Therefore, presence of dioxygen prevents protein aggregation (Schuessler and Schilling 1984). These results are in accordance with the reaction mechanism given by Denk and Schnabel (1982) which describes a sequence of reactions leading to fragmentation. It begins with the attack of OH$^\bullet$ radicals on the biomolecules (PH) (reaction 4.6), and continues with reaction 4.7 yielding peroxyradicals. The biomolecular reaction 4.8 of the peroxyradicals is rate determining and leads to oxyradicals, which finally decompose into fragments (F$_1$+F$_2$).

$$PH + HO^\bullet \rightarrow P^\bullet + H_2O \qquad\qquad (4.6)$$

$$P^\bullet + O_2 \rightarrow PO_2{}^\bullet \qquad\qquad (4.7)$$

$$2\,PO_2{}^\bullet \rightarrow 2PO^\bullet + O_2 \qquad\qquad (4.8)$$

$$PO^\bullet \rightarrow F_1 + F_2 \qquad\qquad (4.9)$$
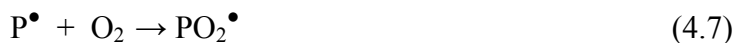
The biomolecules radicals react with formation of aggregates only if oxygen is not present (Denk and Schnabel 1982). Hydroxyl radicals attack on the peptide bonds of the protein yielding carbon-centred radicals. However, the $HO^\bullet$ radicals can also react with aromatic and sulphur-containing amino acid residues with rate constant of about $10^{10}\,M^{-1}s^{-1}$ (Davies, Gilbert et al. 1993). A BSA molecule contains two tryptophan residues, the indole rings of these residues are one of the sites in protein which can be destroyed by oxidation. The reaction of $HO^\bullet$ radicals and oxygen leads to indole ring opening, when protein fragmentation occurs (Schuessler and Schilling 1984).

According to these results, exposure of BSA proteins to irradiation in the presence of dioxygen, results in formation of $BSA^\bullet/BSAOO^\bullet$ protein radicals which react relatively rapidly with ascorbate in solution. Miyazaki and his colleagues reported that the rate constant for the reaction of ascorbate with albumin radicals in the protein coils was measured as $0.014\,M^{-1}s^{-1}$, which is much smaller than the reported rate constants (10^6-10^{10} $M^{-1}s^{-1}$) for the reaction of ascorbate with radicals in an dilute aqueous solution (Miyazaki, Yoshimura et al. 1995). The less efficient oxidation of ascorbate by $BSA^\bullet$ compared to protein peroxyl radicals suggests that $BSAOO^\bullet$ radicals could be the significant source of radicals inducing the ascorbate oxidation.

4.3.1.2 LZ-derived protein radicals

Lysozyme is a small enzyme consists of single polypeptide chains of 129 amino acids cross-linked by four disulphide bridges (Canfield and Liu 1965; Dizdaroglu, Gajewski et al. 1983). In 1983, a paper reported on the radiation-induced formation of α-amino-n-butyric acid, *allo*-threonine, *o*- and *m*-tyrosine, 3-hydroxytyrosine and 2-hydroxytyrosine in lysozyme in N_2O-saturated aqueous solutions (Dizdaroglu, Gajewski et al. 1983). The hydroxyl radicals attack lysozyme and produce intermediate radicals which further on disproportionation would yield the products listed. The HO$^\bullet$ radicals abstract an H atom from aliphatic amino acids either the α-carbon carbon (carbon atom between the nitrogen atom and carbonyl carbon atom) or on the side chain. In general, the reaction rate constant for the H-abstraction at the α-carbon atom at aliphatic amino acid in a peptide chain can taken as approximately 5.1×10^8 M^{-1}s^{-1} (Dizdaroglu, Gajewski et al. 1983). There are two intermediate radicals that can be formed following HO$^\bullet$ radicals attack on a Thr in lysozyme, and these radicals disproportionate and repair to *allo*-threonine. Hydroxyl radicals add to the Trp benzene ring in lysozyme at a diffusion-controlled reaction rate to give hydroxycyclohexadienyl radicals. A hydroxyl radical addition to the aromatic ring of Tyr in lysozyme gives rise to an 3-hydroxytyrosine and 2-hydroxytyrosine intermediate radicals (Dizdaroglu, Gajewski et al. 1983). These intermediate radicals were responsible for the oxidation of ascorbate in my experiments.

In 1993, Franzini *et al.* reported that the hydroxyl radical is a highly-damaging reactive oxygen species for proteins , given its high reactivity and the consequent generation of secondary free radicals (Franzini, Sellak et al. 1993). This study showed that lysozyme was

inactivated by $HO^\bullet$ with a yield of 6.5 mol $HO^\bullet$ /mol lysozyme; moreover, SDS-PAGE showed a loss of native lysozyme (14.4 kDa), the presence of dimer and trimer aggregates and characteristic fragmentation (Franzini, Sellak et al. 1993; Filali-Mouhim, Audette et al. 1997). Pentoxifylline (Ptx, a methylxanthine), uric acid and thymine can scavenge $HO^\bullet$ with high rate constants; however, their effects on $HO^\bullet$-induced alternation of lysozyme were different: uric acid and Ptx prevented aggregation and preserved enzyme activity, whereas thymine preserved activity but did not prevent aggregation. These differences might be related to the formation of reducing secondary radicals, underlining the importance of this mechanism in the effects of scavengers (Franzini, Sellak et al. 1993). Gamma irradiation can modify almost all types of amino acid residues in lysozyme, with little specificity. These workers found 42 moles of modified residues per initial mole of native protein. In contract, only some types of amino acids were modified when lysozyme was exposed to the Fenton reaction. These results confirmed that intramolecular free radical chain reactions played a major role in the oxidative modification of the protein promoted by gamma irradiation (Edwards, Ruiz et al. 2002).

The kinetics of $O_2^{\bullet -}$ reaction with semi-oxidized tryptophan radicals in lysozyme, $Trp^\bullet(Lyz)$, have been investigated by pulse radiolysis. The well-established long range intramolecular electron transfer from Tyr residues to Trp radicals leading to the repair of the semi-oxidized $Trp^\bullet(Lyz)$ and formation of the tyrosyl phenoxyl radical is inhibited by the $Trp^\bullet(Lyz) + O_2^{\bullet -}$ reaction, as is most of the $Trp^\bullet(Lyz) + Trp^\bullet(Lyz)$ reaction. However, the kinetic behaviour of $Trp^\bullet(Lyz)$ suggests that not all oxidized Trp residues are involved in the intermolecular recombination or reaction with $O_2^{\bullet -}$ (Santus, Patterson et al. 2000).

This study indicated that superoxide anion plays a role in a reaction chain leading to radical damage on the protein. This agrees with my study which indicated that superoxide radicals play a significant role of ascorbate oxidation. Of the $LZ^\bullet$ radicals, the $LZOO^\bullet$ had the greatest peroxidant potential for ascorbate oxidation.

Results in Table 3.8 reported that the levels of ascorbate oxidation were similar when caused by $HO^\bullet$, $O_2^{\bullet-}$ or $LZ^\bullet$/ $LZOO^\bullet$ under air. Unlike BSA, the presence of lysozyme made little difference to ascorbate oxidation. However, there was 8.3 μM ascorbate oxidized by $O_2^{\bullet-}$, and 13.4 μM of oxidized by $LZOO^\bullet$ under air. In the absence of dioxygen, $LZ^\bullet$ radicals have a limited ability to oxidize ascorbate, while $HO^\bullet$ can oxidize a significant amount. To conclude, the sequence of the ability in ascorbate oxidation was $LZOO^\bullet > HO^\bullet > O_2^{\bullet-} > LZ^\bullet$. Again, the superoxide anion radicals might play an important role in ascorbate oxidation in my experiments.

4.3.2 Superoxide dismutase treatment

Irradiation of dilute aqueous solutions containing dioxygen leads to the production of both $HO^\bullet$ and $O_2^{\bullet-}$ free radicals in similar amounts. To test if $O_2^{\bullet-}$ radicals contributed most to ascorbate oxidation, we irradiated proteins in the presence of a scavenger enzyme (SOD), known to react rapidly with the $O_2^{\bullet-}$ radicals (Nordberg and Arner 2001). Active SOD treatment during irradiation significantly decreased the yields of oxidized ascorbate (Fig. 3.13), showing that $O_2^{\bullet-}$ radicals were involved in reactions leading to the loss of ascorbate.

4.3.3 Effect of superoxide radicals on ascorbate oxidation

4.3.3.1 Superoxide radicals and carbon dioxide radical anion

The superoxide radical, $O_2^{\bullet-}$, is a highly toxic species that is formed in living organisms (Halliwell and Gutteridge 1999). In addition to metabolic generation, it can be produced by autoxidation of some naturally occurring compounds, such as thiols, oxyhaemoglobin, ferredoxins, adrenaline and tetrahydropteridines (Fridovich 1975). In the presence of dioxygen and 0.1 M sodium formate, $HO^{\bullet}$ reacts to produce $O_2^{\bullet-}$ (G value of $O_2^{\bullet-}$ is 6.0) after gamma irradiation (Davies 1987) (reaction 3.12 and 3.13).

The $CO_2^{\bullet-}$ radical is present in this form throughout most of the pH range and only protonates in strongly acidic solutions (Buxton and Sellers 1973). This radical is also a strongly reducing species, with a redox potential of -2.0 V. It transfers an electron very rapidly to quinones, nitro and nitroso compounds, pyridium, viologen ions, porphyrins, oxygen, and many other organic reducing species so that all the primary radicals of water radiolysis results in eventual reduction of the added solute (Butler and Henglein 1980). However, carbon dioxide radicals can not react with ascorbate. This outcome had been confirmed in my experiments (Fig. 3.16).

4.3.3.2 Superoxide radicals in ascorbate oxidation

Table 4.2 summarizes the results of the effect of superoxide anions on ascorbate oxidation under different expeimental conditions. Three different ways to generate and study only syperoixde anions on ascorbate oxidations are:

I. Under argon, equal amounts of $^\bullet$HO radicals and electrons are generated by water radiolysis. In the presence of O_2 (under air), the electrons are converted to $O_2^{\bullet-}$. Therefore, the difference of oxidized ascorbate levels after irradiation under argon and air indicated the yield of oxidized ascorbate induced by $O_2^{\bullet-}$.

II. Perforimng water radiolysis with sodium formate under air, $^\bullet$HO reacts to produce $O_2^{\bullet-}$ only. Under this condition, ascorbate oxidation is induced only by $O_2^{\bullet-}$.

III: Superoixde anion can be scavenged by SOD. After irradiation, both $^\bullet$HO and $O_2^{\bullet-}$ are produced in the presence of dioxygen. However, the addition of SOD into the solution, only $^\bullet$HO is produced. The difffernce of oxidized ascorbate level in the presence and absence of SOD under dioxygen also provide the the yield of oxidized ascorbate induced by $O_2^{\bullet-}$.

The amounts of oxidized ascorbate produced by superoxide anion were dose-dependent in all three conditions. The yields of oxidized ascorbate induced by $O_2^{\bullet-}$ under different conditions were similar.

Table 4.2 Ascorbate oxidation induced by superoxide anion under different conditions (39.7Gy/min)

Irradiation time (sec)	Oxidized ascorbate (μM)			
	$[Asc]_{air}$-$[Asc]_{Ar}$	$\Delta[Asc]_{formate}$	$[Asc]_{total}$-$[Asc]_{SOD}$	Average
30	4.12	3.59	3.38	3.7
60	5.91	7.61	6.19	6.6
90	7.42	9.62	8.08	8.4
120	8.66	11.94	10.77	10.5

I. $[Asc]_{air}$-$[Asc]_{Ar}$: difference in amounts of ascorbate oxidized under air and argon.
II. $\Delta[Asc]_{formate}$: amount of oxidized ascorbate generated in the presence of formate;
III. $[Asc]_{total}$-$[Asc]_{SOD}$: difference in amounts of ascorbate oxidized with and witout SOD treatment in the presence of dioxygen;
IV: these are different methods to generate superoxide anions during irradiation, which can be used to study the effect of superoxide anions on ascorbate oxidation.

4.3.4 Comparison of ascorbate oxidation by protein radicals under different conditions

Table 4.3 sums up the amounts of oxidized ascorbate formed by various radicals under different irradiation conditions. The major radical involved in ascorbate oxidation in the absence of protein was $O_2^{\bullet-}$, which oxidized the ascorbate directly. In the presence of proteins, the major radical involved in ascorbate oxidation was $HO^{\bullet}$, which reacted with the protein and produced secondary protein peroxyl radicals. The addition of proteins (BSA or LZ) significantly enhanced the level of ascorbate oxidation by about 26-45 %. Hence, protein peroxyl radicals played a major role in ascorbate oxidation. This finding leads to novel approach in studying of $O_2^{\bullet-}$ -mediated protein radical intermediates-induced oxidation.

Table 4.3 The effective radicals involved in ascorbate oxidation under different irradiation conditions

The radicals were identified from the relevant data on radiolysis of water and the concentrations of the reactants in solution.

*Irradiation conditions	Substrate	Oxidized asorbate (μM)	Effective radicals
[1] [Oxidized Asc]$_{O2}$**Error! Bookmark not defined.** − [Oxidized Asc]$_{N2O}$	Asc	6.9	$O_2^{\bullet-}$
	BSA + Asc	12.7	$BSAOO^{\bullet}$
	LZ + Asc	13.4	$LZOO^{\bullet}$
[2][Oxidized Asc]$_{Total}$ **Error! Bookmark not defined.** − [Oxidized Asc]$_{SOD}$	Asc	7.8	$O_2^{\bullet-}$
	BSA + Asc	10.5	$BSAOO^{\bullet}/O_2^{\bullet-}$

* : irradiation dose rate is 104 Gy (120 second irradiation).
1: Difference in amounts of ascorbate oxidized under O_2 and N_2O.
2: Difference in amounts of ascorbate oxidized with and without SOD.

4.4 Ascorbate oxidation by different protein radicals

The sensitivity of different proteins toward ROS depends on factors such as the site where the ROS react, amino acid composition of the protein, localization of the amino acid directly related to the biological activity of the protein, and the presence of scavengers or substances that can repair the damage and other factors (Edwards, Ruiz et al. 2002).

Five proteins studied in my experiment, were BSA, lysozyme, human serum albumin, α-casein, and chymotrypsin. After exclusion of $O_2^{\bullet-}$ contribution (8.0 μM) to ascorbate (Fig. 3.14), the effect of oxidative damage to ascorbate by the hydroxyl radical-generated protein radicals was studied. Ascorbate can be oxidized by different protein radicals with different

effectiveness. Human serum albumin radicals (HSA$^\bullet$) had higher efficiency for ascorbate oxidation than hydroxyl radicals while chymotrypsin radicals (Chy$^\bullet$) were much less effective.

It is well known that the structure and functions of proteins exposed to ROS are altered. Depending on the ROS involved, the nature of the protein and conditions of interaction, protein molecules can undergo oxidative modification of amino acid residues and formation of different protein radicals (Gebicki, Bartosz et al. 1994). However, the biological significance of such observations is difficult to assess. In addition, it has been suggested that the abundant proteins, such as albumin, might be important biological antioxidants counteracting the effects of ROS (Halliwell 1988).

My studies suggest that some of the products of protein modification by ROS are chemically active. One such derivative is the protein radical group attached to the oxidized proteins, which may inactive biological antioxidant such as ascorbate or GSH (Simpson, Narita et al. 1992; Gebicki and Gebicki 1993).

4.5 Ascorbate oxidation by amino acid radicals

4.5.1 Amino acid concentration determination

The concentration of amino acid in these experiments was determined by the reaction rate with HO$^\bullet$ radicals. The reaction rate ratio of AA to ascorbate was kept at over 20 : 1. This result ensures 95 % of hydroxyl radicals preferably react with AA rather than ascorbate in

the solutions. Thus, this design can be used to investigate the reactions between different amino acid radicals and ascorbate.

4.5.2 Amino acid radicals

The oxidation of amino acids by ionizing radiation leads to the formation of oxidized by-products. It has been established that all amino acids are susceptible to oxidation by $HO^\bullet$ (Davies 1987). Dioxygen plays important role of determining the final form of the amino acid radical. In the presence of dioxygen, carbon-centred radicals $(AA^\bullet)$ (Neta, Simie et al. 1969), peroxyl radical derivatives $(AAOO^\bullet)$ and peroxides $(AAOOH)$ are formed. The peroxyl radicals are readily converted to the peroxides by reaction with the protonated form of the superoxide anion radicals or by abstraction of a hydrogen atom from another molecule (Stadtman and Levine 2003). Depletion of dioxygen in reaction mixtures during irradiation suppresses the generation of peroxyl radical and peroxide generation (Stadtman 1993). Many studies have shown that, at neutral pH values and in the presence of O_2, the $HO^\bullet$ radicals cause most of the chemical changes to proteins, the $O_2^{\bullet-}$ radicals being largely unreactive towards amino acids (Bielski and Shiue 1978; Neuzil, Gebicki et al. 1993).

In my experiments, the effect of different amino acid radicals on ascorbate oxidation was studied. The amino acids fell into three groups, those giving no significant amounts of oxidized ascorbate, those giving high yields, and an intermediate group. In the reaction mixtures treated with SOD, $HO^\bullet$ radicals were the only primary radicals formed which induced secondary $AA^\bullet$ radical formation. The amount of oxidized ascorbate produced by

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$O_2^{\bullet-}$ radicals which induced AAOO$^{\bullet}$ as the secondary radicals only can be calculated via [total oxidized ascorbate] - [oxidized ascorbate under SOD-treatment]. The results are shown in Figure 4.3. The aromatic amino acids were not included in this part of study because of the interference by their strong UV absorbance.

Both Cys$^{\bullet}$ and CysOO$^{\bullet}$ were unlikely to react with ascorbate. Val$^{\bullet}$, Ile$^{\bullet}$, Leu$^{\bullet}$ and Gln$^{\bullet}$ radicals had significantly higher capability in ascorbate oxidation than their peroxyl radicals (AAOO$^{\bullet}$), while GlyOO$^{\bullet}$, SerOO$^{\bullet}$, ThrOO$^{\bullet}$, AsnOO$^{\bullet}$ were significantly more efficient in oxidizing ascorbate.

Several observations establish that sulphur centres can act to protect proteins from radical damage by transfer of damage from carbon to sulphur. When hydroxyl radicals react with BSA, the single thiol group appears to act as a radical sink and protects the protein from denaturation and fragmentation through the transfer of damage from a carbon site to the thiol group (Davies, Gilbert et al. 1993). The thiol group of cysteine residues may play an important role in ROS damage to proteins: in the absence of oxygen, aggregation of proteins can occur via the formation of disulfide bonds (Schuessler and Schilling 1984). The loss of activity of certain enzymes is due to oxidation of such residues to sulphoxides or sulphonic acids (Clement, Armstrong et al. 1972). Furthermore, under some circumstances, an electron-transfer mechanism may operate which leads to transfer of the radical species from the initial site of damage to amino acid elsewhere in the protein and in particular to tyrosine, trytophan and cysteine residues, which results in formation of phenoxyl, tryptophanyl and thiyl radicals, respectively (Davies, Gilbert et al. 1993).

In previous studies, exposure of the common amino acids to hydroxyl radicals showed that six of them (glutamate, isoleucine, leucine, lysine, proline, and valine) were peroxidized with similar efficiency to the proteins, whereas the rest were inert or less susceptible (Gebicki and Gebicki 1993). The effect of amino acid hydroperoxides on erythrocyte components was also studied. Amino acid peroxides are relatively stable products of irradiation of amino acid solutions. Interaction of proline, lysine, valine, and leucine hydroperoxides with erythrocyte membranes brought about a decrease of membrane protein -SH group content and of activities of (Na^+, K^+)-ATPase and Ca^{2+} -ATPase, and induced aggregation of membrane proteins, due mainly to the formation of interpeptide disulfides. Interaction of amino acid hydroperoxides with hemoglobin brought about hemoglobin oxidation to methemoglobin. These results indicate that peroxides of amino acid and proteins, which can also be formed under physiological conditions, may be mediators of the cellular action of reactive oxygen species (Soszynski, Filipiak et al. 1996). Only the rate constants of reactions of the aromatic amino acids with ascorbate were studied, because they could be selectively generated in free amino acids, in peptides and in proteins (Josimovic, Jankovic et al. 1993).

At present there is no information on the relative abilities of individual amino acid radicals for ascorbate oxidation. The results in my studies suggest that amino acid may be oxidized by a variety of ROS *in vivo* and their subsequent reactions with a protective agent such as ascorbate may decrease the antioxidant potential of cells and tissues.
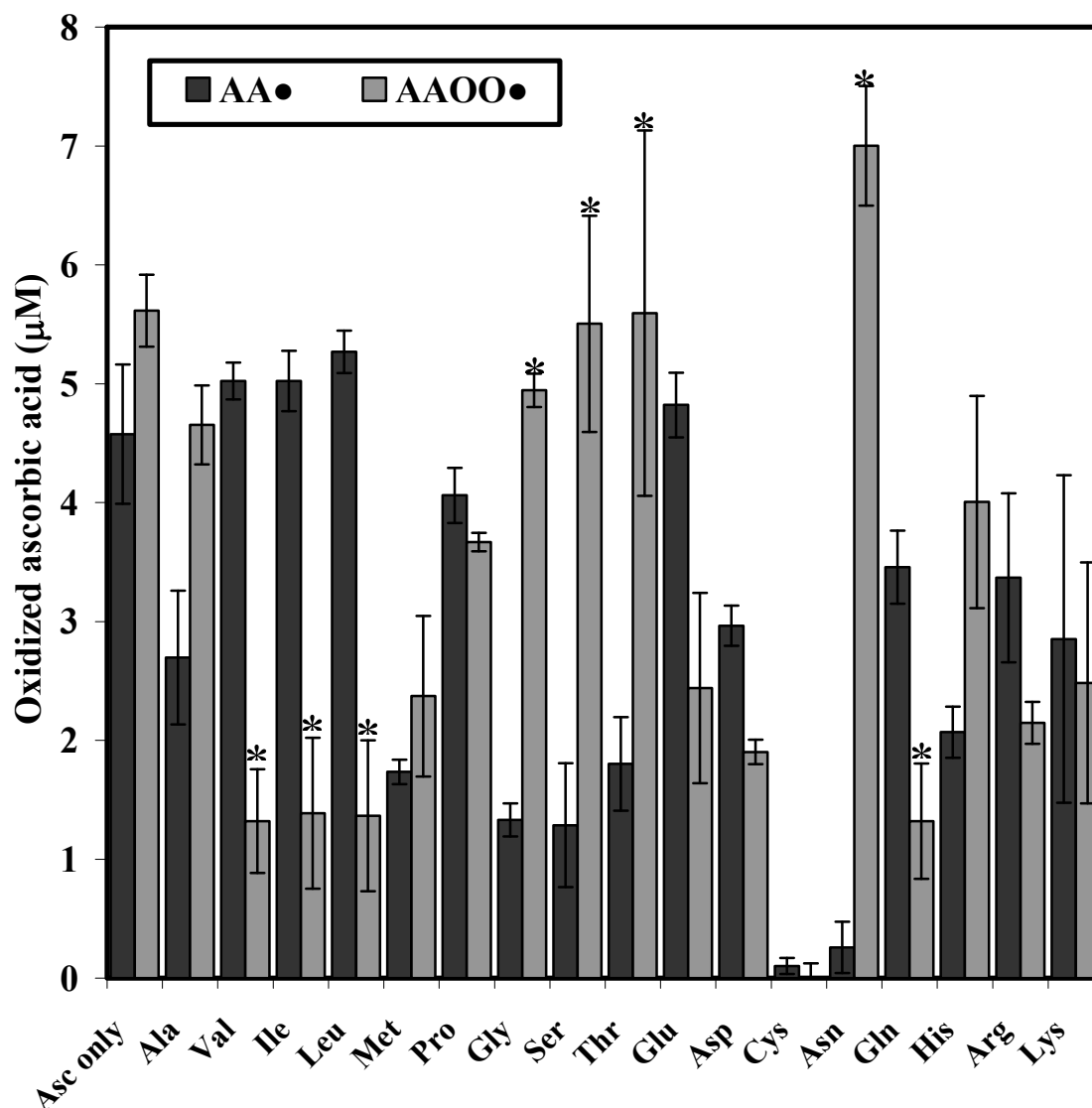
Figure 4.3 The effectiveness of AA$^\bullet$ and AAOO$^\bullet$ radicals in ascorbate oxidation

Sceondary AA$^\bullet$ radicals contributed to ascorbate oxidation only when HO$^\bullet$ radical is the primary radical formed by irradiation and O$_2$ was absent. In the presence of O$_2$ these radicals were converted to AAOO$^\bullet$ radicals. * Indicates statistically significant difference between AA$^\bullet$ and AAOO$^\bullet$ radicals on ascorbate oxidation at $p<0.05$ level.

4.6 Ascorbate oxidation by selected radicals

4.6.1 Ferrozine assay- ascorbate measurement

In 1975, Butts and Mulvihill described an automated method for determining ascorbate in serum and urine by using the reduction of ferric iron by ascorbate and the formation of a colour between the resulting ferrous iron and Ferrozine [3-(2-pyridyl)-5,6-bis(4 phenylsulfonic acid)-1 .2,4-triazine]. This is used to rapidly and simultaneously measure ascorbate in samples and standards and minimize interference from slower reacting substances in the sample. The method is highly precise and specific. Data are also presented on the stability of ascorbate in serum, urine, and aqueous solutions (Butts and Mulvihill 1975; Boyer, Grabill et al. 1988).

The measurement of ascorbate by Vis/UV spectrometry were likely to be affected by the optical absorption of azide radical (274 nm), so that ferrozine assay was chosen to analyze ascorbate in solutions.

4.6.2 Radicals

4.6.2.1 Azide radicals

The azide radical is produced by reaction of the azide ion with $HO^\bullet$ radicals. The reaction of azide with $H^\bullet$ atoms in slightly acidic solution also results in formation of $N_3^\bullet$. The azide radical exhibits moderate optical absorption only in the UV range, with a sharp maximum at 274 nm (Alfassi and Schuler 1985). The azide radical is a strong one-electron oxidant, with a redox potential of 1.3 V. Its oxidation reactions are particularly rapid, even more rapid than the reactions of some stronger oxidants such as $Br_2^{\bullet-}$. This is probably due

to a high self-exchange rate for $N_3^{\bullet}/N_3^{-}$, estimated at 4×10^4 l mol^{-1} s^{-1} (Hewett and Setser 1998). $N_3^{\bullet}$ oxidizes most phenoxide ions and anilines with nearly diffusion-controlled rate constants. It exhibits certain selectivity in its reactions with neutral phenols and with other weaker reductants. It also reacts rapidly with tryptophan, methionine, histidine, phenothiazines, porphyrins, iodide, sulfite, ferrocyanide, etc (Alfassi and Schuler 1985).

The results shown in the Figure 3.21 demonstrated that, hydroxyl radicals were significantly more efficient than azide radicals in ascorbate oxidation. HO$^{\bullet}$ radicals have 2.75 fold higher reaction rate with ascorbate than $N_3^{\bullet}$ radicals (Carr and Frei 1999; Halliwell and Gutteridge 1999) . In my experiments, the same amount of radicals, HO$^{\bullet}$ can oxidize 14.2 μM of ascorbate, but only 9.8 μM oxidized ascorbate was formed by $N_3^{\bullet}$ radicals.

4.6.2.2 LZTrp$^{\bullet}$/ LZTyrO$^{\bullet}$ radicals

Radiation-generated azide radicals can selectively and rapidly oxidizes tryptophan (Trp) residues in many proteins (Land and Prutz 1979; Butler, Land et al. 1982; Weinstein, Alfassi et al. 1991; Bobrowski, Holcman et al. 1997), including lysozyme (LZTrpH), where additional reaction with LZTyrOH makes an approximately 10 % contribution to the disappearance of $N_3^{\bullet}$. Azide radicals can oxidize lysozyme and produce LZTrp$^{\bullet}$ radicals very rapidly, followed by a decay (Nauser, Koppenol et al. 2005). The LZTyrO$^{\bullet}$ radicals did not form immediately, but only in the period corresponding to the decay of the LZTrp$^{\bullet}$ radicals. This is consistent with studies representing intramolecular first-order radical

transfer between these residues in lysozyme and other proteins (Butler, Land et al. 1984; Weinstein, Alfassi et al. 1991; Bobrowski, Holcman et al. 1997).

4.6.3 The effect of lysozyme-induced radicals on ascorbate oxidation

The presence of lysozyme made no difference to hydroxyl radical-induced ascorbate oxidation in the range 20-40 μM ascorbate (Fig. 3.22), which corresponds to the result in Section 4.3.2.2. When both lysozyme and azide were present, the ascorbate appeared to be slightly protected by the protein from azide radical attack. In the absence of azide, ascorbate oxidation was enhanced by addition of lysozyme (Fig. 3.23). The detailed effects of the individual radicals were studied in following experiments.

The amounts of ascorbate oxidized by lysozyme radicals generated by $HO^\bullet$ or $N_3^\bullet$ under N_2O or air were shown in Figure 3.24. $N_3^\bullet$ were generated by steady-state radiolysis in a gamma source of 0.1 M azide solutions with N_2O saturation, as in the fast-kinetic experiments. The $HO^\bullet$ were produced similarly, but without azide. In the absence of azide and dioxygen, the radiation-generated $HO^\bullet$ attacked the lysozyme to produce carbon-centred protein radicals, $LZ^\bullet$ (Franzini, Sellak et al. 1993), which then oxidized the ascorbate (Nauser, Koppenol et al. 2005) (Reaction 4.14 and 4.15).

$$HO^\bullet + LZH \rightarrow LZ^\bullet + H_2O \qquad\qquad (4.10)$$

$$LZ^\bullet + AscH^- \rightarrow LZH + Asc^{\bullet-} \qquad\qquad (4.11)$$

With azide, all $HO^\bullet$ radicals were scavenged to produce $N_3^\bullet$ (Reaction 3.22), followed by formation of $LZTrp^\bullet$ (Reaction 3.23) with some $LZTyrO^\bullet$ generated directly (Reaction

3.24) and by long-range electron transfer from LZTrp$^\bullet$ (Butler, Land et al. 1982). Oxidation of ascorbate can be potentially achieved in reactions 4.12 and 4.13 (Bobrowski, Holcman et al. 1997).

$$LZTrp^\bullet + AscH^- \rightarrow LZTrpH + Asc^{\bullet-} \qquad (4.12)$$

$$LZTyrO^\bullet + AscH^- \rightarrow LZTyrOH + Asc^{\bullet-} \qquad (4.13)$$

Dioxygen is expected to react rapidly with carbon-centred species such as LZ$^\bullet$ (Von Sonntag 1987), but the relevant rate constants have not been measured for protein radicals. The resultant peroxyl radicals are likely to have different reactivities towards AscH and LZ$^\bullet$ and LZTrp$^\bullet$. This was tested by comparing the amounts of ascorbate oxidized by lysozyme radicals generated by radiolysis of solution saturated with N_2O or air (Table 3.16 and Fig. 3.24). With azide, the ascorbate was oxidized only by LZTrp$^\bullet$. No LZTyrO$^\bullet$ was formed by long–range electron transfer from LZTrp$^\bullet$ in the presence of ascorbate, because under the conditions of these experiments reaction 4.12 was faster than the transfer (Table 4.4). Under air, similar amounts of HO$^\bullet$ and superoxide radicals were produced by radiation (Von Sonntag 1987). The former reacted with lysozyme to produce LZ$^\bullet$ and LZTrp$^\bullet$ radicals in the presence of azide, whose most likely date was conversion to the corresponding peroxyl radicals (reaction 4.14). The AscH was then oxidized in reaction 4.15 when azide was absent and in reaction 4.16 in its presence (Nauser, Koppenol et al. 2005).

$$LZ^\bullet/LZTrp^\bullet + O_2 \rightarrow LZOO^\bullet/ LZTrpOO^\bullet \qquad (4.14)$$

$$LZOO^\bullet + AscH^- \rightarrow LZOOH + Asc^{\bullet-} \qquad (4.15)$$

$$LZTrpOO^\bullet + AscH^- \rightarrow LZTrpOOH + Asc^{\bullet-} \qquad (4.16)$$

To sum up, different protein radicals were formed in the different irradiation conditions. Table 4.4 list the published rate constants of reactions of some amino acid and protein radicals with ascorbate and dioxygen.

Table 4.4 Rate constants of reactions of amino acid and protein radicals with selected compounds

Reactant	Radical	Rate constant ($M^{-1}s^{-1}$)	Reference
Ascorbate	Trp$^\bullet$	1×10^8	(Jovanovic and Simic 1985)
Ascorbate	Trp$^\bullet$	9.3×10^7	(Hoey and Butler 1984)
Ascorbate	LZTrp$^\bullet$	8.3×10^7	(Hoey and Butler 1984)
Ascorbate	LZTrp$^\bullet$	8×10^7	(Santus, Patterson et al. 2000)
Ascorbate	LZTyrO$^\bullet$	1.1×10^7	(Hoey and Butler 1984)
O_2	Trp$^\bullet$	$<5 \times 10^6$	(Candeias, Wardman et al. 1997)
O_2	Trp$^\bullet$	$< 10^6$	(Jovanovic and Simic 1985)
O_2	TyrO$^\bullet$	$< 1 \times 10^3$	(Jin, Leitich et al. 1993)
O_2	TyrO$^\bullet$	$\leq 2 \times 10^5$	(Cudina and Josimovic 1987)

4.6.3.1 In the absence of dioxygen

In the irradiation experiments, the relative concentrations of the solutes ensured selective formation of primary and secondary radicals. In the absence of azide, over 99 % of the hydroxyl radicals reacted with the 2 mM lysozyme (k =5.0 $\times$ 10^{10} $M^{-1}s^{-1}$) (Halliwell and Gutteridge 1999) with insignificant direct reaction with the 10 µM Ascorbate (k = 1.1 $\times$ 10^{10} $M^{-1}s^{-1}$) (Buxton, Greenstock et al. 1988). The randomly-generated HO$^\bullet$ initially

oxidized the amino acid located at the protein surface to produce carbon-centred protein radicals with additional damage to α-carbon sites on the backbone. However, in the absence of reactive solutes, these radicals can endure a variety of intramolecular reactions depending on their nature and location, including long-range electron transfer to new sites. Thus, the reactions of protein radicals with solutes may not involve the amino acid initially attacked by $HO^\bullet$ (Butler, Land et al. 1982; Weinstein, Alfassi et al. 1991; Bobrowski, Holcman et al. 1997) . For this reason, the identities of the $LZ^\bullet$ radicals generated by $HO^\bullet$ and reacting with GSH could not be determined (Table 3.16). Since under N_2O all the $HO^\bullet$ produced by the 52 Gy radiation dose generated 29.7 μM $LZ^\bullet$ radicals, the 5.7 μM AscH oxidized (Table 3.16) shows that only about 19 % of the protein radicals took part in oxidation. The rest would be expected to decay by chain process leading to less reactive radicals or non-radical products.

When $N_3^\bullet$ was the oxidant, more precise protein radical identification can be made. The 0.1 M azide scavenged $HO^\bullet$ (k =1.2 $\times$ 10^{10} $M^{-1}s^{-1}$) (Halliwell and Gutteridge 1999), eliminating direct reaction with lysozyme. All the $N_3^\bullet$ then oxidized the protein (k =9.6 $\times$ 10^8 $M^{-1}s^{-1}$) (Nauser, Koppenol et al. 2005) not the AscH present at a very low concentration. The results in Table 3.16 show that the 29.1 μM $LZTrp^\bullet$/$LZTyrO^\bullet$ radicals generated oxidized 4.8 μM AscH with efficiency of 17 %. The $LZTrp^\bullet$ reacted simultaneously with ascorbate, transferred electrons to LZTyrOH and also clearly decayed in other reactions, since they contributed only about 50 % to the formation of $LZTyrO^\bullet$ (Bobrowski, Holcman et al. 1997). As the Table 3.16 reported, the 85 % of ascorbate oxidation was carried out by $LZTrp^\bullet$ /$LZTyrO^\bullet$ radicals out of the total $LZ^\bullet$ radicals. The

results demonstrated that the aromatic amino acids in the protein are the preferred sites for free radical location in the absence of dioxygen.

4.6.3.2 In the presence of dioxygen

The amounts of ascorbate oxidized by the protein radicals in the presence of O_2 was significant greater than then in its absence. This reveals that the essential enhancement role of the O_2 element lies in its ability to trap and stabilize radical centres (Table 3.16). Under air, the amounts of oxidized ascorbate formed by lysozyme radicals produced by $HO^{\bullet}$ and $N_3^{\bullet}$ can be enhanced 1.8-1.9 fold in the oxygenated system. When proteins are oxidized by the $HO^{\bullet}$ radicals, the peroxyl radicals produced are precursors of protein hydroperoxides, known to form in lysozyme and other proteins (Simpson, Narita et al. 1992; Gebicki and Gebicki 1993). However, the amounts of AscH oxidized by the protein radicals were astonishingly high and show that not only the peroxyl radicals involved in hydroperoxide formation were effective (Table 3.16). The G value for hydroperoxide groups in lysozyme is about 0.8-OOH per 100 eV of absorbed energy (Gebicki and Gebicki 1993), whereas those of AscH oxidized are closed to 2.0 (Table 3.16). The G-value of 2.7 for the lysozyme radicals initially generated demonstrated virtually 75 % efficiency of the reaction chain: $HO^{\bullet}/N_3 \rightarrow LZ^{\bullet} \rightarrow LZOO^{\bullet} \rightarrow AscH$.

Even thought hydroxyl radical has the ability to generate tryptophan radical, its high reactivity and low discrimination allows the formation of a wide range of initial carbon-centred radicals by abstraction of H-atom from the protein backbone and amino acid side chains and by the addition to the aromatic residues (Davies and Dean 1997). The results in

Table 3.16 confirm that HO$^\bullet$ radicals can generate peroxyl radicals which oxidized ascorbate molecules, or that the HO$^\bullet$ radicals initiated radical chains, resulting in the formation of several radicals in protein molecules. In the case of N$_3$$^\bullet$, no such radical chains have been reported and it appears that every LZTrp$^\bullet$ formed initially reacted to form an LZTrpOO$^\bullet$ which oxidized the ascorbate stoichiometrically. It seems likely that the tryptophan residues acted as major sink for amino acid radicals initially produced by the HO$^\bullet$ at the protein surface (Nauser, Koppenol et al. 2005).

To conclude, this study provided an additional support for the damage-enhancing dioxygen effect well known in radiation biology (Van Sonntag 1987). Here it involved chemically simple systems from complete anoxia to air saturation. Since ascorbate oxidation was enhanced under air, it appears that stabilization of the carbon-centred protein radicals generated in reaction 4.14 can out-compete alternative decay pathways (Nauser, Koppenol et al. 2005).

4.6.4 The effect of chymotrypsin radicals on ascorbate oxidation

The results in Table 3.17 showed that Chy$^\bullet$/ChyOO$^\bullet$ protein radicals have a similar ability to ascorbate oxidation as HO$^\bullet$ radicals under air. However, ChyTrpOO$^\bullet$ radicals have less efficiency in ascorbate oxidation than other radicals. Only 40 % of ascorbate oxidation was produced by ChyTrpOO$^\bullet$ out of the total ChyOO$^\bullet$ radicals. In this case, ascorbate was preferably oxidized by other protein radicals than ChyTrpOO$^\bullet$. This suggests that the protein radical/dioxygen reactions are fast and that the peroxyl radicals formed are relatively stable (Nauser, Koppenol et al. 2005).

4.6.5 Summary

In general, this study supports the hypothesis that proteins are likely to be important intermediates in the transmission of the damaging potential of ROS to other biologically significant molecules. It appears that the most abundant cellular ROS targets, the proteins, can act as agents of damage rather than as protective molecules neutralizing the effect of ROS. Discovery of compounds able to inhibit reactions of protein radicals in living organisms may therefore lead to the improvement of many biological outcomes of oxidative stress (Nauser, Koppenol et al. 2005).

4.7 Inhibition of BSA-radical-induced ascorbate oxidation and BSA peroxide formation

4.7.1 Antioxidants on BSA radical-induced ascorbate oxidation

The mechanism of protection of ascorbate from BSA oxidative modification by antioxidants is not known at present. My results shown in Figure 3.26 demonstrate that only TEMPO and TEMPOL were very efficient in inhibition of BSA-radical-induced ascorbate oxidation, while PBN, rutin, Trolox were less protective, and Silibinin showed enhancement.

TEMPO and TEMPOL are piperidine nitoxides which attenuate oxidative damage by detoxification of intracellular radicals such as alkyl, peroxyl, alkoxyl, hydroxyl, superoxide, and thiyl radicals (Damiani, Kalinska et al. 2000). In fact, these compounds are capable of

scavenging carbon-centered radicals at the nitroxide function to give alkylated hydroxylamines, and scavenging of peroxyl radicals on the conjugated benzene ring by a substitution reaction to give nonparamagnetic species (Fig. 4.4) (Beckwith, Bowry et al. 1992). TEMPO and TEMPOL couple with carbon-centered radicals at an almost diffusion-controlled rate (1×10^9 M^{-1}s^{-1}) (Beckwith and Bowry 1988). It appears that protection is due principally to trapping of these BSA radicals, thereby reducing the possibility of direct attack on ascorbate and of indirect attack via reaction with molecular oxygen to give peroxyl radicals. If only peroxyl radicals were formed and were responsible for the ascorbate oxidation, then protection should have been observed only by the aromatic nitroxides. However, because the aromatic nitroxides also trap oxygen-centered radicals (Damiani, Carloni et al. 1999), they provide an additional protective mechanism in those systems where they are likely to be generated (Damiani, Kalinska et al. 2000). These antioxidants had great potential to scavenge ROS-induced BSA protein radicals and produced unreactive by-products. Eventually, ascorbate oxidation was eliminated.

Fig. 4.4 Scheme showing the scavenging of alkyl (R•) and peroxyl radicals (ROO•) by aliphatic and aromatic nitroxide radicals

(Data from Damiani, E., Kalinska, B., Canapa, A., Canestrari, S., Wozniak, M., Olmo, E. and Greci, L. 2000).

4.7.2 Effect of antioxidants on BSA peroxide formation

4.7.2.1 BSA hydroperoxide formation

Protein hydroperoxides are formed when a hydroxyl or peroxyl radical removes a hydrogen atom from an amino acid side chain. The resulting carbon-centred radical reacts with oxygen and hydrogen ions to form a hydroperoxide on the amino acid residue (Gebicki 1997). Though relatively stable, protein hydroperoxides readily react with DNA, oxidizing nucleotides (Luxford, Morin et al. 1999), and forming protein–DNA cross-links (Gebicki and Gebicki 1999). Protein hydroperoxides also oxidize cellular thiols (Soszynski, Filipiak et al. 1996) and consume the key cellular antioxidants ascorbate and glutathione (Simpson, Narita et al. 1992). Protein hydroperoxides have been shown to form on various proteins, plasma proteins, lipoproteins and cellular proteins in living cells (Gieseg, Pearson et al. 2003).

Many techniques have been developed for the detection and quantitation of hydroperoxides, but all have significant shortcomings. Tri-iodide assay has the advantages of good specificity and exact 1 : 1 stoichiometry between the amount of peroxide reacting and iodine produced, allowing quantitation of the hydroperoxide (Gebicki and Gebicki 1993). However, the disadvantages are: the need to exclude oxygen from the reaction and the interference by other oxidants and reductants which are capable of reacting with I^- or I_2 (Jessup, Dean et al. 1994).

FOX assay based on alterative chemistry has been recently applied to a variety of oxidized biological materials. Xylenol orange (XO) is known as a good chelator and quantitative

indicator of a wide range of ions, including iron. It binds to Fe^{3+}, but not Fe^{2+} at low pH to give a colored complex. This reaction is not sensitive to oxygen. In FOX method, the hydroperoxides react with an excess of Fe^{2+} at low pH in the presence of the dye xylenol orange and the amount of Fe^{3+} generated is measured as the Fe–XO complex in the visible absorbance range (Gay, Collins et al. 1999; Gay and Gebicki 2002; Gay and Gebicki 2003).

$$ROOH + 2Fe^{2+} + H^+ \rightarrow ROH + 2\ Fe^{3+} + OH^- \qquad (4.17)$$
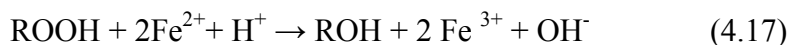
This technique is reasonably sensitive, requires no expensive equipment, has fair specificity for the hydroperoxyl group and is, most importantly, quantitative (Gebicki, Gill et al. 2002).

Dilute aqueous solutions of BSA gave positive tests (FOX assay) for peroxides after exposure to reactive oxygen species (Kouoh, Gressier et al. 1999). In 1993, Gebicki and Gebicki reported that for every 2.8 $HO^\bullet$ radicals formed, 1.16 BSA peroxide groups were produced, provided that the protein concentration was sufficient to scavenge all of the $HO^\bullet$. The solutions were buffered with phosphate, because it is unrecative toward $HO^\bullet$ and $O_2^{\bullet-}$ (Gebicki and Gebicki 1993). In my experiments, bovine serum albumin (BSA) interacted with reactive oxygen species (ROS) and produced peroxides in a radiation dose-dependent manner. According to my results, the yield of BSA peroxides was relatively low compared to previous studies.

4.7.2.2 Antioxidants

Gallic acid [3,4,5-trihydroxybenzoic acid, $C_6H_2(OH)_3COOH$] (Fig. 4.5) and its derivatives are widely present in the plant kingdom and represent a large family of plant secondary polyphenolic metabolites and hence natural antioxidants (Lu, Nie et al. 2006; Madlener, Illmer et al. 2006). It is obtained from nutgalls and other plants or by the hydrolysis of tannic acid with sulfuric acid. Gallic acid (GA) has two functional groups in the same molecule, hydroxyl groups and a carboxylic acid group. They can yield numerous esters and salts including digallic acid which is formed by the reaction of two molecules of gallic acid with one another (Aruoma, Murcia et al. 1993). These polyphenol compounds exhibit different hydrophobicity and can pass through liposome membrane (Lu, Nie et al. 2006). GA derivatives have also been found in many phytomedicines with a number of biological and pharmacological activities, including scavenging of free radicals (Kanai and Okano 1998; Dwibedy, Dey et al. 1999), inducing apoptosis of cancer cells (Sakagami, Satoh et al. 1997; Serrano, Palacios et al. 1998; Saeki, Yuo et al. 2000), inhibiting squalene epoxidase (Abe, Seki et al. 2000) and interfering with the signal pathways involving Ca^{2+} and oxygen free radicals (Sakaguchi, Inoue et al. 1998; Inoue, Sakaguchi et al. 2000; Sohi, Mittal et al. 2003).

As shown in Figure 3.28, BSA peroxide formation can be significantly reduced by the addition of gallic acid, with Trolox and PBN less effective. In 2003, Sohi et al. reported that the protection provided by gallic acid was due to its direct action in the scavenging of free radicals as it was found to be a stronger antiradical than trolox, a water- soluble analogue of vitamin E (Sohi, Mittal et al. 2003). This study corresponded to the result I

obtained in my experiment. Therefore, these results reveal that gallic acid with high antioxidant activity and appropriate hydrophobicity is generally quite effective in preventing ROS-induced protein peroxide formation.
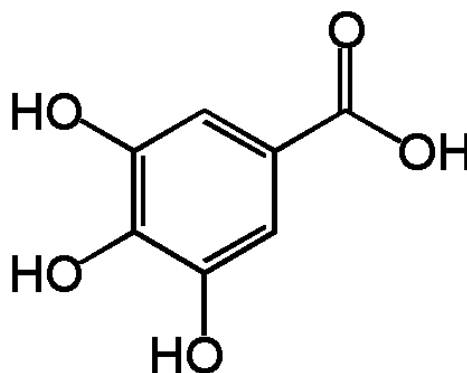
Fig. 4.5 Chemical structure of gallic acid

(From: Madlener, S., Illmer, C., Horvath, Z., Saiko, P., Losert, A., Herbacek, I., Grusch, M., Elford, H. L., Krupitza, G., Bernhaus, A., Fritzer-Szekeres, M. and Szekeres, T., 2006).

SECTION (II) <u>CELL CULTURE STUDIES</u>

4.8 Effect of gamma irradiation-induced oxidative damage to HL-60 cells

Radiation–induced cell damage can occur by direct of indirect energy transfer. Approximately 80 % of the radiation energy deposited within cells is in the radiolysis of water, since it is the major constituent of cells. It has been established that the indirect pathway of radiation damage is predominant and involves generation of primary and secondary radicals (Shankar, Kumar et al. 2003). Ionizing irradiation induces damage mainly due to hydroxyl radicals generated inside the cells, within close proximity to important biomolecules (Yamaguchi, Uchihori et al. 2005).

Most studies assumed that the deleterious effects of ionizing radiation are mostly caused by the direct damage to DNA, either by direct energy deposition or by hydroxyl radicals generated in close proximity. However, several lines of evidences have now appeared that challenge this theory. In 1996, Deshpande *et al.* reported that a greater number of sister chromatid exchanges (SCEs) occurs in cells crossed by alpha particles than theoretically expected by the number of particles traversing their nuclei. The results showed that an extranuclear target through which the alpha particles passed caused DNA damage, as detected by SCE analysis. An extranuclear compartment as a target for alpha particles may have significant contribution to the SCEs and also important implications for the susceptibility of lung cells to the DNA-damaging effects of alpha-particle (Deshpande, Goodwin et al. 1996). In 1998, Lorimore *et al.* demonstrated that alpha-particles induce

chromosomal instability as was confirmed in the descendants of non-irradiated stem cells, suggesting an unexpected interactions between irradiated and non-irradiated cells (Lorimore, Kadhim et al. 1998). In addition, Wu and his colleagues investigated the effect of targeted cytoplasm irradiation on un-irradiated DNA, suggesting that long-lived free radicals or ROS formed in cytoplasm could migrate to the nucleus and induce oxidative damage to the DNA (Wu, Randers-Pehrson et al. 1999). DNA is a vital molecule whose integrity must be maintained, but it is believed to be a secondary rather than a primary target of RS, principally because it is protected *in vivo* by the associated proteins (Ljungman 1991; Caraceni, De Maria et al. 1997; Begusova, Eon et al. 2001; Du and Gebicki 2002).

Recently, the strong evidence became available suggesting that proteins are significant targets for ROS *in vivo*. Increased levels of protein carbonyls and nitrated amino acids, common process of ROS reactions, were found in early stages of exposure of different biological systems to ROS with the antioxidants present unable to prevent the process (Smith, Carney et al. 1991; Stadtman and Oliver 1991; Caraceni, De Maria et al. 1997; Ciolino and Levine 1997; Reinheckel, Nedelev et al. 1998; Bruce, Fu et al. 1999; Merker, Sitte et al. 2000; Beal 2002). Importantly, elevated levels of protein oxidation products were measured in tissues of aged humans and animals, as well as in those suffering from Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, advanced atherosclerotic plaques, and diabetes, leading to the concept that changes in function may be related to progressive oxidation of critical proteins (Beal 2002). While this demonstrates that ROS can cause oxidative damage to proteins *in vivo,* for many years proteins were not

considered to be significant targets of ROS (Halliwell 1988), because there was no evidence of damage by the altered proteins to other cell components. This was provided by the discovery of formation of reactive hydroperoxide groups in proteins attacked by biologically significant ROS (Simpson, Narita et al. 1992). Subsequent work showed that the altered proteins can damage DNA, enzymes, antioxidants, lipids, generate further reactive free radicals and modulate apoptosis. It is now clear that oxidized proteins are a new form of ROS (Gebicki 1997). Proof of formation of protein peroxides *in vivo* was found in cells exposed to reactive oxygen species, in advanced atherosclerotic plaques and in LDL (Dean, Fu et al. 1997; Fu, Fu et al. 1998; Gieseg, Duggan et al. 2000). Alternatively, protein peroxides maybe formed as a result of interaction with hydroxyl radicals, and protein derived radicals may also cause damage to important biomolecules such as DNA (Gebicki 1997).

4.8.1 Effect of irradiation on cell viability in different media

The short time effect of irradiation on cells had been studied in my experiments. To determine the viability of the cells during a short time high dose irradiation, trypan blue and MTT assay were used. As shown in Table 3.30 and Figure 3.31, exposure of cell suspensions to gamma dose rate at 900 Gy, cell viability was significantly reduced from 100 % to 35 % in HEPES buffer. However, cell viability maintained above 80 % in RPMI-1640 medium. Clearly, the loss of cell viability in the irradiated group was most pronounced in cells suspended in HEPES buffer.

Viability assays measure the percentage of a cell population that is viable. This is generally accomplished by a dye exclusion method, where cells with an intact membrane are able to exclude the dye while cells without an intact membrane take up the coloring agent. The dye used for exclusion stain is usually trypan blue but erythrosin and naphthalene black have also been used. A dye uptake stain can be used to measure viability as well. In this case, the dye is normally taken up by viable cells but not by the non-viable cells (Freshney 1994). MTT assay is a standard colorimetric assay for measuring cellular viability. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] assay, first described by Mosmann in 1983, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. Lysis of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The colour can then be quantified using a simple colorimetric assay. The results can be read on a multiwell scanning spectrophotometer (Mosmann 1983). Both methods are based on the intact cell membrane as the index of cell viability, with the MTT additionally providing evidence of mitochondrial activity.

Previous studies reported that radiation dose of hundreds of Grays certainly will inhibit cell viability (Olive, Frazer et al. 1993). However, the cell viability in my experiments showed only slight decrease in RPMI-1640 medium after high dose irradiation. The difference might be caused by the assay time, which in my experiments was completed immediately

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rather than after incubation. In my study, the trypan blue assays was carried out straight after the exposure of irradiation, while the MTT assay requires a four hour incubation time to form crystal. In RPMI-1640 medium, the cell viability differed by 12 % between the trypan blue assay (91 %) and the MTT assay (79%) (Table 3.19). The result suggested that the incubation time after irradiation could affect the cell viability.

Even thought about 900 Gy radiation dose was used in the experiments, based on the volume occupied, the dose per cell was only 2 Gy, which is relatively low. The biological effects of ionizing radiation are mediated through the generation of highly reactive ROS, which also include hydroxyl radicals, superoxide ions, peroxyl radicals and hydrogen peroxide (Martinez, Pennington et al. 1997). These species are produced rapidly under irradiation and react with the molecules in close proximity (Halliwell and Gutteridge 1999). RPMI-1640 medium is a relatively complex solution, containing essential and nonessential amino acids, glucose, vitamins and salts (Freshney 2000). The HEPES buffer used here consist of HEPES, NaCl, $CaCl_2$, $MgCl_2$, and DTT (Guaiquil, Farber et al. 1997). The differences in the viability of cells irradiated in RPMI-1640 medium and HEPES buffer is likely to be due to better recovery in the former medium. Earlier studies have showed that damage to cell proteins by ROS is independent of the composition of the medium, so that the cells were not moderated by the extracellular radical scavengers.

4.8.2 Formation of protein peroxides

The two most probable groups of biomolecules that can be peroxidized by ROS are lipids and proteins. In 2005, Du and Gebicki elucidated that no lipid peroxides were detected in

organic solvent extracts of the cells, even after very high doses of radiation. Since all low molecular weight cell components were removed in the PCA precipitation step of G-PCA-FOX assay, all the peroxides form cells were protein peroxides (Du and Gebicki 2004). The confirmation of the nature of the species formed in γ irradiated cells before any detectable lipid peroxides provided a possible mechanism that could involve radiation-induced bystander effect (Ward 2002), since many protein peroxides can diffuse in cells (Gebicki 1997), can be the source of new free radicals, and induce damage to DNA (Gebicki and Gebicki 1999; Luxford, Morin et al. 1999).

The formation of protein peroxides in γ irradiated cells may also help to explain the oxygen effect during irradiation (Von Sonntag 1987). Hydroxyl radicals produced during irradiation lead to the formation of the carbon centred radicals in targeted proteins. In the presence of oxygen, the protein peroxyl radicals are formed and then the hydroperoxides by hydrogen abstraction (Gebicki 1997). Du reported that cells irradiated in petri dishes produce more protein peroxides than those irradiated in centrifuge tube (unpublished work). This result confirmed the damage effect of oxygen, since petri dishes have (16 fold) larger surface area than centrifuge tube and thus can facilitate the diffusion of oxygen to cells. Protein peroxides formed in solution have a long half-life at room temperature (Gebicki and Gebicki 1993), while those formed in U937 cells remained stable for the first 3 h of incubation in RPMI-1640 media at 37 $^{\circ}$C (Gieseg, Duggan et al. 2000).

If the protein peroxides formed in irradiated cells result from the action of hydroxyl radicals generated in close proximity to protein, there should be no difference between cells

irradiated in HEPES buffer and RPMI-1640 medium (radiation dose rate: 900 Gy). The results shown in Table 3.20 verified this assumption, and suggest that low molecular organic components could not protect the cell proteins from oxidation. It appears likely that protein peroxides are generated by radiation within the cells, making the composition of the medium relatively irrelevant. Obviously, the organic solutes in the cell media were unable to scavenge the HO$^\bullet$ radicals responsible for protein peroxidation or to generate secondary reactive products which can enter the cells and initiate protein peroxidation. The only effective radicals were therefore those formed within the cells or within a few nanometres of the cell surface (Du and Gebicki 2004).

Some studies have shown that irradiation of medium (serum-free) alone could produce active species which may interact with serum components and induced sister chromatid exchanges (SCEs) in un-irradiated cells (Lehnert and Goodwin 1997). Cells exposed to irradiated medium containing 8 % serum have a small increase in mutant fraction (Zhou, Suzuki et al. 2002). The main component of the RPMI-1640 media responsible for the generation of ROS was riboflavin, but tryptophan, tyrosine, pyridoxine, and folic acid enhanced the effect of riboflavin (Grzelak, Rychlik et al. 2001). All studies indicated that irradiated culture medium alone could produce active species, which cause damage to un-irradiated cells, but it is not known whether these species include amino acids and protein peroxides.

It has been shown that the long half-life radicals, rather than short half-life ones, are generated in irradiated cells and able to induce mutation and morphological transformation

(Yoshimura, Matsuno et al. 1993; Koyama, Kodama et al. 1998). It is possible that these radicals formed in cells are protein-derived, which can also induce protein-to-protein damage transfer, protein chain-oxidation, and also antioxidant-derived radical formation (Irwin, Ostdal et al. 1999). Østdal et al. reported that incubation of Fe (III) myoglobin with H_2O_2 in the presence of bovine serum albumin can give rise to albumin-derived radicals as a result of radical transfer from myoglobin to BSA. These protein-to-protein radical transfer reactions may have important consequences for understanding protein oxidation in biological systems (Ostdal, Andersen et al. 1999). This might provide the evidence why cytoplasmic irradiation could cause nuclear DNA damage (Wu, Randers-Pehrson et al. 1999).

4.8.3 Effect of irradiation dose on the formation of protein peroxides

As shown in Figure 3.33, the amounts of protein hydroperoxides increased in a radiation dose-dependent manner, and the rate (0.02-0.03 μM/min) of peroxide formation was much lower in the initial 15 min irradiation than later (0.35-0.38 μM/min). This outcome might be due to the protection by thiol groups in cells. In the first fifteen minutes, protein thiols might act as radical sinks preventing the formation of protein hydroperoixdes (Platt and Gieseg 2003). After that, protein peroxides were accumulated in the cells at accelerated rate (Du, J., private communication).

4.9 Effect GSH depletion and repletion on protein peroxide formation in HL-60 cells

GSH is a tripeptide of glycine, glutamate (glutamic acid) and cysteine (Balendiran, Dabur et al. 2004; Wu, Fang et al. 2004). Among the biological mechanisms which protect cells (e.g. against oxidative stress), thiol groups, and GSH in particular, have a central role in the endogenous defence of the body (Shan, Aw et al. 1990). Glutathione (γ-glutamylcysteinylglycine) functions in catalysis, metabolism, transport, and reductive processes and in protection of cells by destruction of free radicals, reactive oxygen intermediates, and other toxic compounds of endogenous and exogenous origin (Meister, Anderson et al. 1986). Glutathione is ubiquitous in animals, plants, and microorganisms, and being water soluble is found mainly in the cell cytosol and other aqueous phases of the living system. Glutathione often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants (Kosower and Kosower 1978; Lomaestro and Malone 1995). Glutathione is fundamental as a reducing agent in the metabolism of hydrogen peroxide, organic peroxides and free radicals (Pastore, Federici et al. 2003); it thus provides protection to tissues from oxidative damage. GSH is the most effective antioxidant occurring naturally in the cells. GSH also makes major contributions to the recycling of other antioxidants that have become oxidized (Lomaestro and Malone 1995; Masella, Di Benedetto et al. 2005). GSH reacts rapidly and nonenzymaticly with hydroxyl radicals, peroxynitrite, and hydroperoxides. This thiol-containing reductant also maintains so-called thiol-enzymes in their catalytically active form, and maintains vitamins C and E in their biologically active forms (Griffith and Mulcahy 1999). All glutathione peroxidases reduce hydrogen peroxide, alkyl and organic hydroperoxides at the expense of

glutathione (Brigelius-Flohe 1999), but not hydroperoxides generated in large protein (Gebicki, Gill et al. 2002). GSH can also be oxidized and consumed by protein hydroperoxide group (Simpson, Narita et al. 1992). Moreover, GSH has been shown to prevent apoptosis and to maintain viability in cells lacking oncogene *bcl-2* (Kane, Sarafian et al. 1993), a gene whose product is able to block the onset of apoptosis through a radical-scavenging mechanism. GSH's antioxidant defences are therefore of the greatest importance (Raggi, Mandrioli et al. 1998).

The rate of GSH synthesis is determined and regulated by the availability of substrates, predominantly cysteine. GSH also functions as a storage and transport form of cysteine (Meister, Anderson et al. 1986). Cysteine is normally derived from the diet and protein breakdown, and in the liver from methionine via the transsulfuration pathway of homocysteine (Griffith and Mulcahy 1999; Lu 1999). Cysteine, the sulfhydryl form, is predominant inside the cell; whereas its disulfide form, cystine, is predominant outside the cell. Cysteine can autoxidize to cystine in the extracellular fluid; once it enters the cell, cystine is rapidly reduced to cysteine (Anderson 1997).

GSH depletion is commonly carried out by L-Buthionine-sulfoximine (BSO) treatment, which is an inhibitor of GSH synthesis that acts by selectively inhibiting the enzyme, γ-glutamylcysteine synthetase, responsible for combining glutamate and Cys to form γ-glutamylcysteine (Plummer, Smith et al. 1981). The administration of BSO to mice leads to depletion of 10-20 % GSH in most tissues, while 95 % of GSH can be depleted in most cell

cultures (Griffith and Mulcahy 1999). Therefore, BSO can induce oxidative stress in a cell by irreversibly inhibiting γ-glutamylcysteine synthetase (Reliene and Schiestl 2006).

N-acetylcysteine (NAC) is a thiol-containing compound which nonenzymatically interacts and detoxifies reactive oxygen species. NAC can effectively protect human bronchial fibroblasts against the toxic effects of tobacco smoke condensates and the isolated perfused lung against the glutathione GSH-depleting effect of tobacco smoke. NAC is also known to reduce the reactive oxygen intermediate hydrogen peroxide and protect against the toxic effects of H_2O_2. In vivo studies, however, demonstrated that the direct scavenging effect of ROS by NAC does not seem likely. A more relevant mechanism in vivo for the protective effect NAC against toxic species may be due to NAC acting as a precursor of GSH facilitating its biosynthesis. GSH will then serve as the protective agent and detoxify reactive species both enzymatically and nonenzymatically (Moldeus, Cotgreave et al. 1986). N-Acetyl cysteine, is readily taken up by cells and is used for GSH synthesis (Reliene and Schiestl 2006).

It has been shown that free thiols inhibit PrOOH formation with both AAPH peroxyl radicals and X-ray generated hydroxyl radicals (Platt and Gieseg 2003). It is well established that GSH readily reduces ethyl, lipid and other hydroperoxides via react with and break down them. However, nothing is known about its ability to inhibit formation of protein hydroperoxides in cells. In the present investigation, the intracellular GSH level in HL-60 cells was modulated by *N*-acetylcysteine (NAC), a GSH precursor, and buthionine sulfoximine (BSO), a specific GSH synthesis inhibitor. The data from these experiments

provide direct evidence showing that GSH is involved in protein peroxide generation in cells, either by inhibiting its formation or by reduction.

4.9.1 Effect of gamma irradiation on intracellular GSH levels in HL-60 cells

Previous studies showed that intracellular GSH levels are correlated with radiation sensitivity of cells, GSH levels decline only in radiation-sensitive cells after irradiation but not in radiation resistant ones (Vlachaki and Meyn 1998). The cell radiation response could be determined by the level of GSH. GSH-depleted cells remained more sensitive to irradiation, and increased GSH levels provided radioprotection (Clark, Epp et al. 1986; Vos and Roos-Verhey 1988; Vos and Roos-Verhey 1988). Earlier studies also indicated a relationship between cellular GSH content and radiation response (Revesz 1985).

In order to study the relationship between intracellular GSH levels and protein peroxide formation, Figure 3.33 and 3.38 were combined as Figure 4.6. As shown in Figure 4.6, during the first 15 minutes there was a slightly faster loss of GSH in irradiated cells (170 μM / min). The levels of GSH continued to decrease during the rest of 20 min irradiation, but at a slower rate (60 μM / min). In contrast, there was a slow formation of protein peroxides in the first 15 min of irradiation and the sharp rise at 20 min irradiation. This is consistent with the concept that after intracellular GSH dropped to a certain level, the ability of cells to remove protein radicals was compromised, which results in the accumulation of the hydroperoxides.

The GSH level reduction in the first 15 min radiation is likely to be the result of the direct reaction with short-lived and organic radicals. The slow and steady consumption of GSH at later stages may results only from a variety of reactions with compounds generated by irradiation. Cells incubated in Hank's balanced salt solution could cause the loss of intracellular GSH by lacking GSH precursors (Clark, Epp et al. 1986). However, the level of GSH in non-irradiated cells was maintained 100 % of controls in my experiments (data not shown) when the cells were suspended in RPMI-1640 medium. In this system, there were cysteine and other GSH precursors available, so that the export of GSH through its transporter via γ- glutamyl cycle could be balanced by re-synthesis (Lu 1999). To sum up, GSH plays an important role in regulation of protein peroxide formation in cells.

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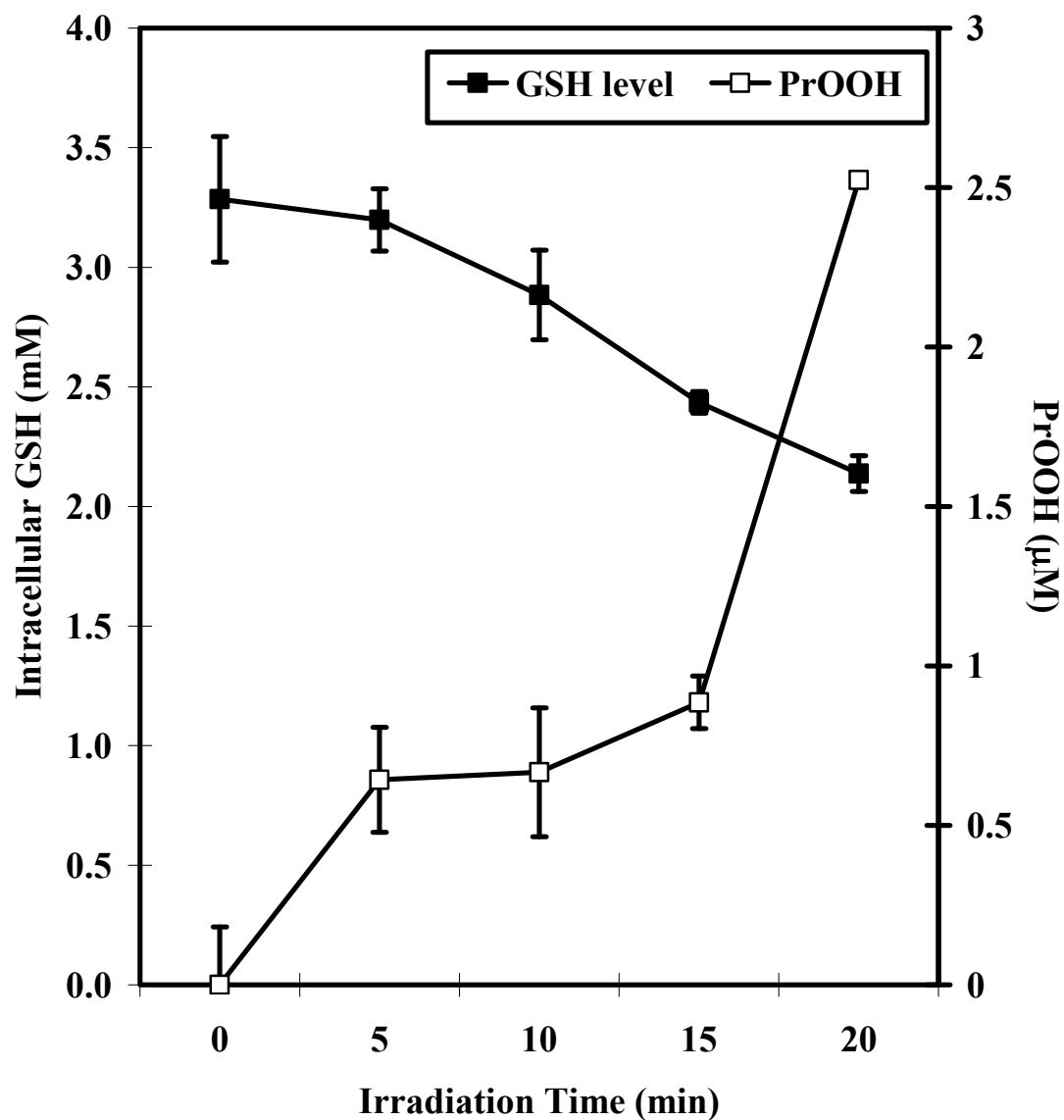
Figure 4.6 Comparison of intracellular GSH levels and protein peroxide formation in HL-60 cells after gamma irradiation in RPMI-1640 medium

This figure is obtained by combination of Figure 3.33 and 3.38. Intracellular GSH was measured by Ellman's assay. Protein peroxide concentrations of cells were measured by G-PCA-FOX assay. The data points were derived from triplicate experiments, with error bars obscured by the symbols.

4.9.2 Effect of GSH depletion in HL-60 cells

4.9.2.1 Effect of BSO on intracellular GSH level and cell viability in HL-60 cells

BSO has been used to deplete cells of glutathione (Meister 1985) and to obtain HL-60 cells containing different intracellular concentrations of GSH. To achieve the maximum GSH depletion without loss of viability, the incubation conditions had to be assessed. As shown in Figure 3.39, when HL-60 cells were incubated with a range of BSO concentrations for 24 h in RPMI-1640 growth medium, the intracellular GSH levels were reduced significantly, 100 μM BSO treatment offered up to 85 % depletion, with no further depletion with higher concentrations of BSO. BSO showed no cellular toxicity under the experimental conditions studied, and cell viability as assessed by MTT assay was always greater than 95 % (Fig. 3.40). Incubation with BSO for 16-48 h can significantly deplete intracellular GSH with negligible toxicity in mammalian cells (Coe, Rahman et al. 2002; Wu, Fang et al. 2004). Different cell lines might have different sensitivities to BSO treatment, thus, GSH depletion can be restricted through the time of exposure and the concentration (Clark, Epp et al. 1986; Bellomo, Vairetti et al. 1992). The level of GSH was significantly decreased in a radiation dose-dependent manner in HL-60 cells (Fig. 3.41).

4.9.2.2 Effect of BSO on protein peroxide formation in γ-irradiated cells

Depletion of glutathione (effectively accomplished by inhibition of its synthesis) increases sensitivity to radiation, while increased cellular levels of glutathione protects cells against radiation. The depletion of intracellular GSH by BSO has been shown to enhance cell radioresponse (Clark, Epp et al. 1986), to enhance radiation-induced tyrosine phosphorylation (Tuttle, Horan et al. 1998), and to increase cellular mutagenic response to

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cytoplasmic irradiation (Wu, Randers-Pehrson et al. 1999). Previous studies indicated that GSH has an important role in keeping intracellular redox balance; it is implicated in the cellular defence against xenobiotics and naturally occurring deleterious compounds, such as free radicals and hydroperoxides (Pastore, Federici et al. 2003). However, it is still unclear whether GSH acts as an antioxidant in response to ROS-induced random damage to cell components, especially proteins, during irradiation.

This study has shown that γ irradiation induced formation of protein peroxides in cells, in parallel with the loss of intracellular GSH (Fig. 4.6). In 1998, Vlachaki and Meyn proposed that many radicals contributed directly to the depletion of GSH during irradiation (Vlachaki and Meyn 1998). The persistent loss of GSH after irradiation maybe due to its enzymatic or non-enzymatic reaction of protein peroxides (Fu, Gebicki et al. 1995; Gebicki, Gill et al. 2002). Pre-incubation of cells with BSO results in 150 % increase in peroxide formation in cell irradiated for 20 minutes (Fig. 3.42), indicating that GSH depletion compromised cellular ability to inhibit the formation of protein peroxides and enhanced radiation-induced oxidative damage to proteins. While these results do not prove that GSH is the main or only molecule protecting cells from protein peroxidation, it clearly plays an important role in this process.

4.9.3 Effect of GSH repletion in HL-60 cells

4.9.3.1 Effect of NAC on intracellular GSH level and cell viability in HL-60 cells

N-Acetylcysteine (NAC) is a thiol-antioxidant precursor of glutathione (GSH) used in human therapy (Delneste, Jeannin et al. 1997). It can scavenge free radicals such as hydroxyl radicals, hydrogen peroxide, hypochlorite and peroxynitrite (Moldeus, Cotgreave et al. 1986; Aruoma, Halliwell et al. 1989). It has been shown that if NAC enters a cell, it will be rapidly hydrolyzed and the cysteine processed further in various ways (Berggren, Dawson et al. 1984; Sjodin, Nilsson et al. 1989). By entering the GSH synthetic pathway, cysteine enhances GSH synthesis (Moldeus, Cotgreave et al. 1986). In 1998, Tuttle *et al.* found that incubation of CHO-K1 cells with 20 mM NAC for 1 h led to a twofold elevation in GSH levels (Tuttle, Horan et al. 1998), and preincubateion of HepG$_2$ cells with 5 mM NAC for 24 h resulted in 70 % GSH rise.

As shown in Figure 3.43, when HL-60 cells were incubated with a range of NAC concentrations for 3 h in RPMI-1640 serum-free medium, the intracellular GSH levels were increased significantly; 1 mM NAC treatment offered up to 42 % enhancement. NAC also showed no cellular toxicity under the experimental conditions studied, and cell viability as assessed by MTT assay was always greater than 95 % (Fig. 3.44). Since intracellular L-cysteine levels are consistently and substantially lower than levels of L-glutamate and glycine, L-cysteine availability is generally limiting cellular GSH level (Griffith and Mulcahy 1999). As a drug, NAC administration can increase intracellular GSH level (Cotgreave, Sandy et al. 1987; Cotgreave 1997). As shown in Figure 3.45, the level of GSH

was significantly decreased in a radiation dose-dependent manner in HL-60 cells, especially in 20 min irradiation.

4.9.3.2 Effect of NAC on protein peroxide formation in γ-irradiated cells

Some clinical observations have been published on the radioprotection after the use of NAC (Kim, Baker et al. 1983; Solen 1993). NAC has been shown to effectively reduce ROS and other oxidant species. The hydrogen atom in the thiol (ie, -SH) group of many sulfur-containing antioxidants can act as an electron donor for neutralizing free-radicals. The reaction with these oxidants results mainly in the formation of NAC disulphide (Olsson, Johansson et al. 1988). In 1998, Tuttle and his colleagues suggested that the effects of NAC on radiation-induced signal transduction are due to its ability to alter the intracellular reducing environment, and are not related to direct scavenging of ROS (Tuttle, Horan et al. 1998). The amount of intracellular and intra-nuclear GSH increased with prolonged incubation time with NAC. An increase in intracellular GSH concentration was correlated with an increase in radiation resistance in some cell lines (Vos and Roos-Verhey 1988).

As shown in Figure 3.46, when the intracellular GSH levels of HL-60 cells were increased by pre-incubation with NAC, the amounts of protein peroxides formed decreased as measured immediately after irradiation, especially after 20 min irradiation. Thus, the results provided additional evidence that the formation of protein peroxides can be inhibited by intracellular GSH. This study elucidated the first time that NAC can inhibit protein peroxides formation via an increasing intracellular GSH levels.

4.9.4 Comparison of the effect of GSH depletion and repletion in γ-irradiated HL-60 cells

Exposure to radiation produces hydroxyl radicals, the most reactive radical known. Studies show that GSH acts as a radiation protector. The radiosensitivity of cells depends on intracellular GSH levels (Tuttle, Horan et al. 1998). Through the accumulated evidence, it must be concluded that glutathione plays a key role in cellular resistance against oxidative damage, not only as a free radical scavenger, but also for its emerging role as protein-bound glutathione (Pastore, Federici et al. 2003). As shown in Figure 3.47, the intracellular GSH level was reduced in a radiation-dose dependent manner. The protein peroxide formation was controlled by the amount of GSH in cells (Fig. 3.48). It appears that intracellular GSH may be able to react protein carbon-centred radicals generated by gamma irradiation thus inhibit the formation of protein peroxides. Alternatively, peroxides can be rapidly reduced by GSH in cells.

Besides, *in vitro* study, we suggested that the thiols such as Cys can effectively remove $O_2^{\bullet-}$ or $^{\bullet}OH$, and the S-centred Cys radical reacted with O_2 to generate the unreactive $CysOO^{\bullet}$. The thiol group on protein can react with radicals and form unreactive protein thiol peroxides which reduce the protein peroxide –induced damage in cells.

4.10 Inhibition of protein peroxidation by ascorbate in gamma-irradiated HL-60 cells

Ascorbate is a radical trapping antioxidant and is considered one of the most efficient antioxidants (Frei, Stocker et al. 1990). Some studies have shown that HL-60 cells accumulate high concentrations of ascorbic acid by a complex mechanism involving the facilitated transport of dehydroascorbate (DHA) down a concentration gradient through facilitative glucose transporters, followed by the reduction of DHA to ascorbic acid and the intracellular trapping of ascorbic acid (Vera 1994; Vera 1995; Welch, Wnaf et al. 1995). Exposure of cells to ionizing radiation can cause protein peroxide formation (Gebicki and Gebicki 1993). In 1992, Simpson and his colleagues discovered that protein hydroperoxides can consume key cellular reductants, such as ascorbate and glutathione (Simpson, Narita et al. 1992). Since antioxidants have been shown to protect against radiation-induced peroxide formation, in this study we have evaluated the putative protective effect of ascorbate against radiation-induced production of peroxides in the HL-60 cells. We addressed the question as to whether ascorbate loading of the cells could reduce protein peroxide formation induced by gamma-irradiation.

4.10.1 The effect of ascorbate on cell viability

In 1996, Menoto *et al*. studied the effect of ascorbate on cell growth using primary cultured hepatocytes and chondrocytes elicited from guinea pigs and six kinds of cell lines derived from the tissue and blood of mammals. They cultured cells in medium supplemented with or without ascorbate at various concentrations for 24 and 48 h and reported that different cell species have varying sensitivities to ascorbate. HL-60 showed the growth inhibition at

higher concentrations of ascorbate in medium among the observed cells, which were damaged by the exposure to different concentrations of hydrogen peroxide. These results indicate that the concentration of ascorbate in medium required to inhibit cell growth depends on the activity of catalase in the cells (Nemoto, Otsuka et al. 1996). In another study, ascorbate also potentiated proliferation of HL-60 cells in serum-limiting media. Dehydroascorbate does not affect cell growth (Navas and Gómez-Díaz 1995). The rate of cell growth was not influenced by different culture medium (Young and Tsao 1992). Ascorbic acid treatment has also been reported to increase the survival of irradiated cells in vitro and in vivo (Yasuda, Tada et al. 2004).

In the present study, we examined the cytotoxic effect of ascorbate on HL-60 cells. Data from this study clearly showed that ascorbate has no effect on cell viability in both control and irradiated cells (Fig. 3.49 and 3.50) in RPMI-1640 medium. These results again confirmed that only the radicals formed within the cells were effective in oxidizing the cell proteins.

4.10.2 Intracellular accumulation of ascorbate in HL-60 cells

Like many other cells lines, HL-60 cells are unable to transport the reduced form of ascorbic acid, AscH, but only the oxidize form of ascorbic acid, DHA (Vera 1994). The accumulation of ascorbic acid in HL-60 cells is a process that can be dissociated into at least two components with characteristic kinetics. The first step consists of the facilitated transport of DHA, followed by its reduction and intracellular accumulation as ascorbic acid. Transport was mediated by GLUT1, the member of the family of facilitative glucose

transporters expressed in the HL-60 cells (Vera 1995). In the second step, once transported, DHA was immediately reduced intracellularly to ascorbate (Welch, Wnaf et al. 1995). The results of the uptake of DHA assay indicated that the ascorbic acid rapidly accumulated in the cells, reaching a maximum at 30 min (Witenberg, Kalir et al. 1999). Since less than 1 pmol of ascorbic acid can be detected by using the high-performance liquid chromatography with coulometric electrochemical detection, this assay provides significantly greater sensitivity than nearly all of the currently available procedures (Washko, Hartzell et al. 1989).

The average concentrations of ascorbate in human blood plasma range from 20-200 μM (Welch, Wnaf et al. 1995), and ascorbate levels in human tissues are generally far higher. They are particularly high in cornea, lens, and aqueous humor of the eye (up to 1.5 mM) and in adrenal and pituitary glands (up to 2.5 mM). Brain, heart, liver, spleen, kidneys, and pancreas also contain high concentrations of ascorbate (up to 0.8 mM) (Frei, Stocker et al. 1990).

In my studies, HL-60 cells were treated with ascorbic acid and ascorbate oxidase for 30 min. The quantitative HPLC analysis was performed to measure ascorbic acid which has accumulated in the cells. The HL-60 cells accumulated high (mM) intracellular concentrations of ascorbic acid, which agrees with previous studies. Washko *et al.* indicated that activated neutrophils have a 10-fold or more increases above their endogenous millimolar ascorbic acid at precisely the time they require protection from the oxidants they are producing (Washko 1993).

In my study, the intracellular ascorbate accumulation occurred in a dose-dependent manner. The intracellular ascorbate levels were significantly lowered by irradiation, demonstrating its consumption by the radiation-generated radicals, especially at higher ascorbic acid loading dose (Fig. 3.53 and Table 3.23). Many studies have reported that ascorbic acid in cells can provide antioxidant protection against oxidative damage induced by the radiation. Ascorbic acid itself can be oxidized and produced more stable radicals which can be recycled by to ascorbic acid via an enzymatic system (Arrigoni and De Tullio 2002).

4.10.3 The effect of ascorbate in protein peroxide formation

It is well documented that reactive oxygen species are capable of damaging many cellular biomolecules, including protein, and induce the formation of protein peroxides. In the case of HO$^{\bullet}$ radical randomly-generated by gamma irradiation, protein peroxides are produced in a variety of cell types (Du and Gebicki 2004). Recently, it was shown that cells with a higher intrinsic level of peroxide production showed a higher sensitivity to induction of apoptosis (Lotem, Peled-Kamar et al. 1996). In our study, we have shown that exposure of HL-60 cells to increasing levels of gamma irradiation resulted in elevated intracellular concentrations of peroxides (Fig. 3.33).

In parallel experiments, we have shown that exposure of HL-60 cells containing increasing concentrations of intracellular ascorbic acid to gamma irradiation resulted in reduced formation of protein peroxides (Fig. 3.56 and Table 3.24). We consider that ascorbic acid, being an antioxidant, neutralizes precursor protein radicals of peroxides and decreases the intracellular oxidative level, and thus protects cells from oxidative induced damage. The

results here at the tissue culture level are consistent with previous test tube studies that have shown that ascorbic acid can be consumed by the protein radicals and protein peroxides formed in the irradiation. We therefore conclude that ascorbate protects HL-60 cells against radiation-induced protein peroxide formation, although the mechanisms of protection must still be elucidated.

4.11 General conclusion and further work

Ascorbate was efficiently oxidized by protein radicals generated in reaction of HO$^\bullet$ in the presence of O$_2$. Any contribution of the radical-radical dimerization of the HO$^\bullet$ can be ignored because of the low steady-state radical concentrations and the much higher concentrations of solutes used in this study. Both carbon-centred and peroxyl protein radicals oxidized ascorbate. Amino acid radicals were also able to oxidize ascorbate. The damage-enhancing dioxygen effect was confirmed in our studies. Protein radical / dioxygen reactions are fast and the peroxyl radicals formed are relatively stable. Oxidation was enhanced in the presence of oxygen, suggesting that stabilization of the carbon-centred protein radicals generated by dioxygen can out-compete alternative decay pathways. The results suggest that protein radicals formed *in vivo* may lower the antioxidant potential of the organisms by the destruction of ascorbate. HL-60 cells loaded with ascorbic acid reduced protein hydroperoxide formation induced by γ-irradiation, suggesting that the vitamin can protect cells from oxidative stress by repairing their precursor protein radicals. However, this results in loss of ascorbate, which must be replenished for continuing protection of the cells.

Studies of the formation and reactions of proteins oxidized by ROS and their potential significance *in vivo* are still in the early stages. However, as the potential of activated proteins to cause significant downstream damage to some vital molecules became clearer, the prospect of practical application of this knowledge to alleviate the outcomes of oxidative stress in living organisms began to emerge. For this, much needs still to be done in discovering conditions preventing excessive oxidation of proteins and in developing methods of interrupting the pathway of biological damage initiated by ROS and other reactive species at the level of proteins. It seems that the formation of protein radicals and protein peroxides is a general phenomenon following oxidative stress, and that the reactions of protein radicals and peroxides with cellular thiols and ascorbate may contribute to the shift of cellular redox balance. Therefore, the new approach may involve a new range of antioxidants specifically able to repair protein radicals, or to maintain the normal antioxidant machinery by outcompeting their destruction by the activated proteins. There is clearly still a long way to go, but there is also enough experimental evidence to suggest that studies of the consequences of protein oxidation and its prevention should be profitable directions for continuing research.

In the future, this approach could explore a major new direction in studies of the mechanism of biological damage by ROS and its prevention in biological systems. Also it can provide the basic information of trials designed to protect animals, and then humans, from oxidative stresses associated with aging, diseases and cancer.

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