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**Antioxidant potential of selected dietary
and medicinal agents;
implications for improving *in vivo*
antioxidant status**

by

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Doctor of Philosophy
in The Hong Kong Polytechnic University

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LIST OF PUBLISHED AND PRESENTED WORK FROM STUDY

Journal Articles

1. Benzie, I.F.F., Szeto, Y.T. Total antioxidant capacity of teas by the ferric reducing / antioxidant power (FRAP) assay. *Journal of Agricultural and Food Chemistry* (1999), 47(2), 633-636.
2. Benzie, I.F.F., Szeto, Y.T., Strain, J.J., Tomlinson, B. Consumption of green causes rapid increase in plasma antioxidant power in humans. *Nutrition and Cancer* (1999), 34 (1), 83-87.
3. Szeto, Y.T., Benzie, I.F.F. Simultaneous measurement of total antioxidant capacity and ascorbic acid of common fruits and vegetables. *Journal of Food Science* (submitted, Mar 1999).

Conference paper

4. Szeto, Y.T., Benzie, I.F.F., Tomlinson, B. *In vitro* antioxidant activities of various types of Chinese tea. *Atherosclerosis* (1998), 136 (Suppl), S77.
5. Benzie, I.F.F., Szeto, Y.T., Strain, J.J., Tomlinson, B. Bioavailability of antioxidant power in green tea. *Atherosclerosis* (1998), 136 (Suppl), S35.
6. Benzie, I.F.F., Szeto, Y.T., Tomlinson, B., Strain, J.J. Plasma antioxidant power increases after ingestion of green tea. *Journal of the Hong Kong College of Cardiology* (1998), 6 (1), 63.
7. Benzie, I.F.F., Szeto, Y.T., Tomlinson, B. Antioxidant potential of lipid lowering drugs *in vitro*. *Journal of the Hong Kong College of Cardiology* (1998), 6 (1), 51.
8. Benzie, I.F.F., Szeto, Y.T., Tomlinson, B., Strain, J.J. Bioavailability of tea antioxidants. Abstract presented at The Association of Clinical Biochemists National Meeting, 11-15 May 1998, UK
9. Benzie, I.F.F., Szeto, Y.T. Green tea and *in vivo* antioxidant status: post ingestion changes in plasma ferric reducing / antioxidant power (FRAP) confirm absorption and systemic distribution of polyphenolic tea antioxidants. Abstract presented at The Nutrition Society Irish Section Annual Meeting, 22-24 July 1998, UK

10. Benzie, I.F.F., Szeto, Y.T. Antioxidant potential and ascorbate content of fresh fruits and vegetables. Abstract presented at The Second International Conference on Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease, 24-27 June 1998, Helsinki, Finland.
11. Benzie, I.F.F., Szeto, Y.T., Tomlinson, B., Strain, J.J. Drinking green tea leads to a rapid increase in plasma antioxidant potential. In: Kumpulainen, J.T., Salonen, J.T. eds. Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease. Cambridge: Royal Society of Chemistry 1999, pp 280-282.
12. Szeto, Y.T., Benzie, I.F.F. Fruits and vegetables as sources of antioxidant potential. *Journal of the Hong Kong College of Cardiology* (1999), 7 (1), 60.
13. Benzie, I.F.F., Tomlinson, B., Chung, W.Y., Szeto, Y.T. Atorvastatin treatment-related changes to *in vivo* oxidant: antioxidant balance. Abstracted accepted for presentation at the 71st European Atherosclerosis Society Meeting, Greece, May 1999.
14. Benzie, I.F.F., Szeto, Y.T., Tomlinson, B. Assessment of *in vitro* genoprotective or genotoxic effects of dietary antioxidants using the comet assay. Abstract accepted for presentation at the 17th International & 13th European Congress of Clinical Chemistry & Laboratory Medicine, 1st International Congress of Clinical Molecular Biology (CMB), 31st National Congress of the Italian Society of Clinical Biochemistry and Clinical Molecular Biology, 6-11 June 1999, Firenze, Italy.

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ABSTRACT

The main purpose of the study was to assess the potential for dietary agents, such as teas, fruits and vegetables, and selected drugs and medicinal agents, to increase the antioxidant status of the body, reduce oxidative stress and thereby, lower risk of diseases associated with increased oxidative stress.

The specific aims of the study were:

1. To measure and compare the *in vitro* total antioxidant (reducing) power of various types of teas, drugs, fruits and vegetables, and selected medicinal agents.
2. To measure the bioavailability of the antioxidant power in selected beverages with high *in vitro* total antioxidant (reducing) power by monitoring post-ingestion changes in plasma and urine.
3. To measure the potential protective and/or genotoxic effects of selected antioxidants, e.g. tea polyphenolics and ascorbic acid (vitamin C).

In vitro total antioxidant capacities and ascorbate content of more than 30 varieties of fruits and vegetables, including several Chinese fruits and vegetables, were studied by the FRASC assay. The database of antioxidant capacity and ascorbic acid level of fruits and vegetables is being developed and will hopefully form the basis of a long-term systematic study. Also, selected hypoglycaemic and lipid lowering drugs were tested for antioxidant power.

The antioxidant power of different types of teas (*Camellia sinensis*) was also investigated. The ferric reducing/antioxidant power (FRAP) assay was used to measure the total antioxidant power of freshly prepared infusions of 29 types of teas including black, oolong and green teas. Results showed that different teas had widely different *in vitro* antioxidant power and that the antioxidant capacity was strongly correlated with the total phenolics content of the tea.

Green tea was found to contain very high antioxidant power and was selected for the bioavailability study. Post-ingestion changes in the plasma and urine antioxidant power of 10 adults were measured at timed intervals. Results showed that absorption of tea antioxidants was rapid, with a peak increase in plasma around 4% at 40 minutes. Urinary excretion of absorbed polyphenolic antioxidant was also fast, peaking at 60-90 minutes.

Purified polyphenols from tea and wine were purchased and tested for DNA protective and damaging effects on human lymphocytes. The single cell gel electrophoresis test (Comet assay) was used for determining DNA damage in individual cells before and after polyphenol treatment. DNA protective effect was tested after polyphenol incubation followed by oxidative stress induced by H_2O_2 treatment. Uric acid and antioxidant vitamins (ascorbic acid, α -tocopherol) were included for comparison. Results showed that quercetin, caffeic acid and α -tocopherol, were protective against oxidant stress. Ascorbic acid at low concentration ($< 200 \mu\text{mol/l}$) showed a trend of protection but this did not reach a statistically significant level. Catechin, epicatechin and catechin gallate showed no apparent DNA protective or damaging effect. Uric acid and ascorbic acid at high

concentration ($> 200 \mu\text{mol/l}$), and epigallocatechin, epigallocatechin gallate, resveratrol, green tea, black tea and Lingzhi at all concentrations tested caused increased damage.

Results indicate that various plant-based foods and some medicinal agents have high antioxidant potential. Results have shown that at least some of the antioxidant power in polyphenolic rich dietary agents is absorbed and enters the plasma. Results also show, however, that DNA damage may be increased in the presence of some antioxidants. Further study is needed to assess the clinical effect of increased antioxidant status.

CHAPTER INTRODUCTION AND AIMS OF 1 PROJECT

Scope And Background Of Research

Oxidative stress-related changes to protein, lipid and DNA may lead to increased risk of diseases such as coronary heart disease and cancer. Oxidative stress is caused by reactive oxygen and nitrogen species (ROS, RNS, “free radicals”). These species are formed during normal aerobic metabolism and also as a consequence of cigarette smoking, ionizing radiation, inflammatory processes and post-ischaemic reperfusion. Many epidemiological studies have shown a strong inverse relationship between antioxidants and risk of coronary heart diseases and cancer. Increased intake of antioxidants may also slow the onset of cataract and improve the status of patients with Parkinson’s disease.

The body is equipped with various antioxidants to oppose the effects of ROS and RNS. However, if antioxidants are depleted for any reason, for example, dietary deficiency, oxidative stress will increase, and tissue damage may follow quickly. In contrast, if antioxidant defence status can be increased, oxidative changes may be prevented or slowed and risk of disease lowered. Many members of the *in vivo* antioxidant defence team are endogenous compounds. However, dietary input is the sole source of some antioxidants, including vitamin C and vitamin E. Other possibly important dietary antioxidants include flavonoids and polyphenols, found in plant-based foods and in beverages such as red wine and tea. In addition, various Chinese herbal remedies are reported to have antioxidant activity. The bioavailability and action of these plant-derived polyphenolic

antioxidants, however, is not yet established. If the relative antioxidant properties and bioavailability of antioxidants in tea, wine, and Chinese herbs can be measured and compared, simple inexpensive and palatable dietary strategies for increasing *in vivo* antioxidant defence status can be planned and their effectiveness in lowering oxidative stress assessed.

The primary aim of this study, therefore, was to assess the potential for selected dietary agents to lower oxidative stress and, thereby, decrease risk of diseases associated with increased oxidative stress. This potential being assessed by measuring the ferric reducing / antioxidant power (FRAP) assay value. A secondary aim was to perform preliminary studies of possible pro-oxidant effects of selected antioxidants using a recently established test of oxidative DNA damage and use this test in assessing pro-oxidant and antioxidant effects of selected dietary agents.

CHAPTER 2 LITERATURE REVIEW AND BACKGROUND OF STUDY

Introduction

When life first appeared on earth, there was no or little gaseous oxygen. Those primitive living organisms acquired energy from glycolysis rather than aerobic respiration. At that time, life forms did not confront strong oxidants and high PO_2 and oxidative stress did not exist (Halliwell & Gutteridge, 1989; Ho *et al.*, 1995).

Photosynthesis of green plants changed the composition of the atmosphere dramatically and the atmosphere became oxidising. As the content of O_2 increased, many defenceless primitive organisms probably died of oxygen toxicity. To increase the chance of survival, the remaining species could either avoid contact with molecular oxygen by staying away from aerobic environment, or they had to adapt to the aerobic condition by developing antioxidant defence systems to protect against O_2 toxicity. O_2 toxicity is caused by the oxidation of organic molecules leading to damage to biological systems such as the central nervous system and respiratory system. The rates of these oxidation reactions are slow at normal temperatures but rates can be increased by enzymes or heat. Antioxidant defences may prevent oxidation reactions by removing or inactivating oxidising species or catalysts of oxidation. Alternatively antioxidants may be preferentially oxidised in order to protect biomolecules such as proteins, DNA, carbohydrates and lipids (Benzie, 1996a; Halliwell, 1997).

Antioxidants can be classified in terms of their location, action, solubility and source (Halliwell, 1994a). Diet is a source of certain important antioxidants, and increased intake of dietary antioxidants may improve antioxidant defence status, and hence may lower the risk of certain oxidative stress-related diseases (Strain & Benzie, 1998a). In this chapter, types and sources of reactive species, types and sources of antioxidants, oxidative stress, antioxidant defence and the association of oxidative stress with disease will be reviewed. In addition, the potential role of dietary agents in lowering risk of chronic diseases associated with increased oxidative stress, measurement of antioxidant capacity and possible means of improving antioxidant status by dietary means will be discussed.

Reactive Species

A free radical has been defined as any species capable of independent existence that contains one or more unpaired electrons (Halliwell & Gutteridge, 1989). The unpaired electron usually increases the chemical reactivity of an atom or molecule (Halliwell, 1994). Examples of free radicals are superoxide ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), the glutathione radical (GS^{\cdot}), trichloromethyl radical (CCl_3^{\cdot}) and nitric oxide ($^{\cdot}NO$) (Halliwell, 1993). Some reactive species which are nonradicals, but which may, nevertheless, lead to oxidative damage, include singlet oxygen ($^1\Delta gO_2$), ozone (O_3), hypochlorous acid ($HOCl$) and hydrogen peroxide (H_2O_2) (Halliwell, 1997). These and other reactive species are listed in Table 2.1.

Table 2.1 Reactive oxygen and nitrogen species of physiological importance
(adapted from Halliwell, 1997)

Reactive oxygen species (radicals): Molecular oxygen (O_2^{\bullet}), Superoxide ($O_2^{\bullet-}$), Peroxyl, (RO_2^{\bullet}), Hydroperoxyl (HO_2^{\bullet}), Alkoxy (RO^{\bullet}), Hydroxyl ($^{\bullet}OH$)
Reactive oxygen species (nonradicals): Singlet oxygen ($^1\Delta gO_2$), Hydrogen peroxide (H_2O_2), Hypochlorous acid ($HOCl$), Ozone (O_3),
Reactive nitrogen species (radicals): Nitric oxide (NO^{\bullet}), Nitrogen dioxide (NO_2^{\bullet})
Reactive nitrogen species (nonradicals): Nitrous acid (HNO_2), Dinitrogen tetroxide (N_2O_4), Dinitrogen trioxide (N_2O_3), Peroxynitrite ($ONOO^{\bullet}$), Peroxynitrous acid ($ONOOH$), Nitronium cation (NO_2^+), Alkyl peroxynitrates ($ROONO$)

Generation of Reactive Oxygen Species (ROS) In Vivo

Oxidation and formation of free radicals cannot be avoided in human aerobic metabolism. Aerobes use oxygen as the final electron receptor during redox-linked generation of energy in the form of ATP. The four electron reduction of O_2 to give two water molecules is a thermodynamically favoured reaction, but the reaction usually proceeds via 1 or 2 electron transfer steps (Ho *et al.*, 1995). Some of the electrons entering the electron transport chain become uncoupled, probably from ubiquinone, and leak out, reacting with O_2 to form $O_2^{\bullet-}$ (Gutteridge & Halliwell, 1994; Winyard *et al.*, 1994). $O_2^{\bullet-}$ is, therefore, a 'normal' by-product of aerobic respiration, and the quantity produced is likely to increase with increased cellular respiration, e.g. during exercise and hypermetabolic states such as thyrotoxicosis (Alessio, 1993).

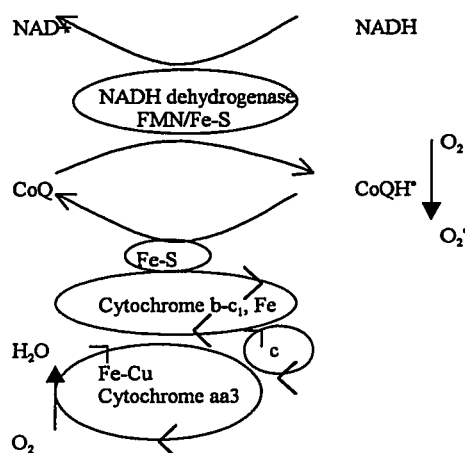


Figure 2.1 Generation of superoxide by the electron leakage from the electron transport chain (adapted from Marks *et al.*, 1996)

Various oxidases and oxygenases use oxygen directly *in vivo*, and this is a further source of ROS. For example, D-amino acid oxidase uses O₂ to oxidise unwanted D-amino acids, with production of H₂O₂, which may produce [•]OH (Gutteridge & Halliwell, 1994). Xanthine oxidase (XO) exists in normal cells mainly in the form of xanthine dehydrogenase (XDH) (Chen *et al.*, 1995). XDH can be transformed to XO, however, by limited proteolysis or oxidation of sulphhydryl groups (Parks & Granger, 1986). XO oxidises xanthine and hypoxanthine to form uric acid with production of H₂O₂ and O₂•⁻. Haemoglobin also generates radicals daily because the Fe²⁺ of the haem group will associate with O₂ with the formation of Fe³⁺ and O₂•⁻ (Gutteridge & Halliwell, 1994).



O₂ is also used to oxidise toxic materials such as drugs, toxins or pesticides by the cytochrome P450 system in body tissues, especially liver (Gutteridge & Halliwell, 1994). This mixed function oxidase system may produce radical species during

'detoxification', e.g. formation of trichloromethyl radical from carbon tetrachloride.

As well as being accidental but avoidable, generation of ROS sometimes is deliberate and useful. Oxygen-dependent mechanisms occur in anti-microbial systems which can be found in the phagocytic vacuole of activated neutrophils and macrophages. The mechanism involves the production of ROS such as $O_2^{\bullet-}$, $^1\Delta gO_2$, HOCl and $^{\bullet}OH$ (Roitt, 1994)(Table 2.2). That this is a beneficial, purposeful process can be exemplified by chronic granulomatous disease (CGD) in which there is minimal or no capacity in phagocytes to generate $O_2^{\bullet-}$ by NADPH oxidase. The characteristic of this disease is that the patient is recurrently infected by catalase positive bacteria (Moslen, 1994).

Table 2.2 Oxygen dependent mechanisms of ROS production in phagocytic vacuoles (adapted from Roitt, 1994)

$NADPH + O_2$	\rightarrow	$NADP^+ + O_2^{\bullet-} + H$
Cytochrome b_{245}		
$2O_2^{\bullet-} + 2H^+$	\rightarrow	$H_2O_2 + ^1\Delta gO_2$
$O_2^{\bullet-} + H_2O_2$	\rightarrow	$^{\bullet}OH + OH^- + ^1\Delta gO_2$
$H_2O_2 + Cl^-$	\rightarrow	$OCl^- + H_2O$
Myeloperoxidase		
$OCl^- + H_2O$	\rightarrow	$H_2O + Cl^- + ^1\Delta gO_2$

NO^{\bullet} is generated continuously in the body by nitric oxide synthase (NOS) from oxidation of L-arginine. NOS is important for vasodilation, neurotransmission and cytotoxic action against foreign organisms (Garrel &

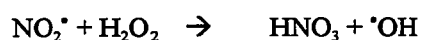
Fontecave, 1995), but NO^\bullet can react with O_2^\bullet to form peroxynitrite (ONOO^\bullet) which is a reactive specie.

Exogenous Sources of Reactive Species

Reactive species can also be found in our environment. Frequent contact with, *in vivo* inhalation, or ingestion of those reactive species may increase oxidative stress. Hence knowing the sources of exogenous reactive species may help us to avoid the damage caused by them.

Cigarette smoke

Cigarette smoke contains a considerable number of reactive species, including ROO^\bullet , NO^\bullet , and may contain H_2O_2 . The gaseous compound NO^\bullet in cigarette smoke can react with O_2 to form NO_2^\bullet (Halliwell & Gutteridge, 1989). NO_2^\bullet is a radical which can attack hydrocarbons with $\text{C}=\text{C}$ double bonds and generate peroxy radicals. Both NO^\bullet or NO_2^\bullet can react with H_2O_2 present in cigarette smoke to produce $^\bullet\text{OH}$, a highly reactive species.



H_2O_2 increases the number of neutrophils in the lung with concomitant increase in oxidant production in the forms of O_2^\bullet and H_2O_2 (Halliwell & Gutteridge, 1989).

Other air pollutants

Combustion of most biological materials and motor vehicle exhausts will produce oxides of nitrogen. Ozone (O_3) is also a common air pollutant with strong

oxidising power. Ozone can lead to stimulation of lipid peroxidation and may deplete some antioxidants such as GSH, vitamins C and E. Sulphur dioxide (SO_2) dissolves in water and can form the sulphite ion (SO_3^{2-}). Sulphite radical ($\text{SO}_3^{\cdot-}$), sulphate radical ($\text{SO}_4^{\cdot-}$) and the peroxysulphate radical ($\text{SO}_5^{\cdot-}$) are known to be produced during sulphite oxidation and the reactivity of $\text{SO}_4^{\cdot-}$ and $\text{SO}_5^{\cdot-}$ are very high (Halliwell & Gutteridge, 1989).

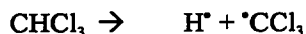
Alcohol

Large doses of ethanol have been reported to decrease the superoxide dismutase (SOD) activity and glutathione (GSH) concentrations of kidney and liver tissues of rats (Halliwell & Gutteridge, 1989). Ingestion of alcohol has been shown to lower the level of antioxidants such as α -tocopherol in rats (Loop *et al.*, 1994). The pathogenesis of liver injury caused by high intake of alcohol may involve the generation of hydroxyethyl radicals during metabolism by P-450 system. The species may be responsible for lipid peroxidation stimulation and liver protein alkylation. The production of H_2O_2 and $\text{O}_2^{\cdot-}$ by cytochrome P450, xanthine oxidase and aldehyde oxidase could also contribute to ethanol-related oxidative damage (Albano *et al.*, 1993).

Others

The painkiller paracetamol has been reported to deplete GSH and to induce lipid peroxidation in the liver of animals (Fischer *et al.*, 1985). Paracetamol is a substrate of the cytochrome P-450 system and its metabolite, paracetamol quinoneimine which is highly reactive, can attack proteins and deplete GSH by reacting with $-\text{SH}$ groups. Carbon tetrachloride and chloroform

under UV irradiation can undergo homolytic fission producing radicals. Generation of $\cdot\text{CCl}_3$ can also occur in the P-450 system (Halliwell & Gutteridge, 1989).



Although UV light does not have energy as high as that of X-rays and γ -rays, UV can cause homolytic fission of H_2O_2 and, therefore, generate hydroxyl radicals, resulting in damage to DNA by single-strand breaks and DNA-protein cross-links (Halliwell & Gutteridge, 1989). This may be important as some parts of the body are exposed to UV light, e.g. skin and eye. Reactive species may also be ingested in food, or formed from dietary agents within the GI tract, such as semiquinone radicals formed from carminic acid (Donnelly & Robinson, 1995; Halliwell, 1995a). Hence, the body is always being exposed to various types of reactive species.

Oxidative Stress

Generation of reactive species exceeding the antioxidant capacity of a biological system leads to oxidative stress. Increased reactive species production in or introduction to the body is likely, therefore, to increase oxidative stress. Oxidative stress is defined as an imbalance in the pro-oxidant-antioxidant equilibrium in favour of the pro-oxidants (Sies, 1993). Oxidative stress can result from either increased production of pro-oxidants or by weakening of antioxidant defence, and this leads to a disturbance of the pro-oxidant-antioxidant steady state (Sies, 1993). An organism may respond to mild oxidative stress by making extra antioxidant defences but severe oxidative stress can cause cell damage or even

death (Halliwell, 1994). Moreover, some antioxidant defence mechanisms cannot be altered from within.

Antioxidant Defence and Repair Mechanisms

Several types of antioxidant defence occur *in vivo*. The first line of defence is prevention antioxidants. Prevention can be achieved by certain enzymes and proteins that suppress the formation of reactive species. The second line is radical scavenging. Some low molecular mass molecules can be used to scavenge ROS, preventing, or at least minimising, oxidative damage (Strain & Benzie, 1998a).

When the above-mentioned antioxidant defence mechanisms cannot cope with oxidative stress, another approach is to repair the damage done by free radicals (Epe, 1993; Niki, 1993; Gutteridge & Halliwell, 1994; Halliwell *et al.*, 1995). Repairing mechanisms are shown in Table 2.3, but will not be discussed further.

Table 2.3 Repair of oxidative damage (adapted from Halliwell *et al.*, 1995a)

Substrate Damaged	Attacked by	Repair System
DNA	$\cdot\text{OH}$, $^1\Delta\text{gO}_2$	Many enzymes (e.g. formamidopyrimidine-DNA glycosylase, endonuclease III & UvrABC excinuclease) recognise abnormalities in DNA & remove them by excision, resynthesis & rejoining of the DNA strand
Proteins	-SH group attacked by many ROS, $\cdot\text{OH}$ attacks many amino acid residues	Methionine sulfoxide reductase can repair oxidised methionine residues. Cellular proteases can destroy damaged proteins.
Lipids	Some ROS ($\cdot\text{OH}$, $\text{LO}\cdot$ & $\text{LOO}\cdot$ but not H_2O_2 & $\text{O}_2\cdot^-$) can initiate lipid peroxidation	Chain-breaking antioxidants remove chain-propagating peroxy radicals. Peroxides from membrane can be removed by certain enzymes (e.g. glutathione peroxidase). Damaged lipids can be released during normal membrane turnover.

Types of Antioxidants

Antioxidants can be divided into different classes such as extracellular or intracellular, exogenous or endogenous, lipophilic or hydrophilic, and preventive, scavenging or chain breaking, enzymic and non-enzymic.

Non-enzymic antioxidants

The category of non-enzymic antioxidants includes the small molecules that react directly with reactive species and disarm them. Some, such as uric acid, can also chelate transition metals, thereby inhibiting metal-catalysed oxidation reactions (Davies, 1995). Selected non-enzymic antioxidants for discussion in the following section are vitamin E, β -carotene, vitamin C, glutathione, uric acid, bilirubin, ubiquinol/ubiquinone, α -lipoic acid and flavonoids (Table 2.4).

Table 2.4 Summary of functions or properties of non-enzymic antioxidants

Vitamin E	major membrane-bound antioxidant and free radical scavenger that can terminate chain reactions among unsaturated fatty acid molecules; lipid soluble, dietary derived
β-carotene	lipid-soluble antioxidant, likely has similar function as vitamin E but unclear, dietary derived
Vitamin C	water-soluble antioxidant, scavenges $O_2^{\cdot-}$, $\cdot OH$, 1O_2 , may regenerate vitamin E and scavenges urate anion radical <i>in vitro</i> , dietary derived
Glutathione	reacts directly with carbon-centred, peroxy, phenoxyl and semiquinone radicals ; endogenous; mainly intracellular
Ubiquinol/ Ubiquinone	lipid-soluble antioxidant; effective antioxidant in membranes, possibly by donating its hydrogen to pro-oxidant
Uric Acid	water-soluble antioxidant; similar function as vitamin C; chelator of transition metals (iron, copper); endogenous
Bilirubin	lipid-soluble antioxidant; scavenges peroxy radical effectively in homogeneous solution or multilamellar liposomes, endogenous
α-lipoic acid	lipid-soluble antioxidant, scavenges, $\cdot OH$, 1O_2 , $HOCl$ and chelates Cu^{2+} , Mn^{2+} and Zn^{2+} in aqueous solution; endogenous and dietary derived

Vitamin E

Vitamin E is a group of compounds which include tocol and trienol derivatives exhibiting the biological activity of α -tocopherol and which have antioxidant activity, stabilize polyunsaturated lipids and lower lipid peroxidation. Humans cannot synthesise vitamin E and must obtain this in the diet (Combs, 1992). Vitamin E includes eight forms: α -, β -, γ - and δ -tocopherols and α -, β -, γ - and δ -tocotrienols. This lipid soluble species is carried in all plasma lipoproteins (Traber, 1996; Sheehy & Morrissey, 1998). Since the tocopherol molecule is carried in LDL and HDL, lipid standardisation is necessary for expressing vitamin E status (Thurnham *et al.*, 1986). Vegetable oil, seeds and grains are important dietary sources of vitamin E. Most of the absorbed tocopherols are transported unchanged to the tissues. The lower limit of serum vitamin E of adults is 11.6 $\mu mol/l$ (Sheehy & Morrissey, 1998). The *in vivo* antioxidant power of vitamin E

results in its oxidation to tocopherylquinone through the semi-stable intermediate tocoperoxyl radical. Each tocopherol molecule can provide 2 electrons but the tocoperoxyl radical is thought to be recycled by reaction with ascorbic acid (Buettner, 1992).



Vitamin E is believed to have a basic function in the maintenance of membrane integrity. Its antioxidant function involves the reduction of carbon-centred free radicals in order to protect unsaturated fatty acid against the harmful reaction of such highly reactive oxidising species. Vitamin E has antioxidant power capable of stopping chain reactions among polyunsaturated fatty acids (PUFAs). Vitamin E scavenges by donating the phenolic hydrogen atom to fatty acyl free radicals (Combs, 1992). The dietary intake of vitamin E mainly consists of γ -tocopherol (Traber, 1996) but *in vivo* the most active and abundant form is α -tocopherol (Stocker & Bowry, 1996) since tocopherol-binding proteins bind to α -tocopherol more effectively than γ -tocopherol (Traber, 1996).

The results of retrospective and prospective studies on vitamin E generally indicate an inverse relationship between cancer incidence and plasma vitamin E level and/or vitamin E intake. Low vitamin E intake or plasma level are also related to higher coronary heart disease risk (Weber *et al.*, 1997; Benzie, 1998; Gey, 1998). Fasting plasma concentration of tocopherol in healthy adults is 1.7-2.4 $\mu\text{mol}/\text{mmol}$ (Total lipid standardised) (Thurnham *et al.*, 1986) and recommended daily allowance (RDA) for adult men is 10 mg or 15 IU (Diplock,

1985; Sheehy & Morrissey, 1998). Intake of > 100 IU vitamin E /day for at least 2 years has been reported to lower risk of CHD (Stampfer & Rimm, 1995).

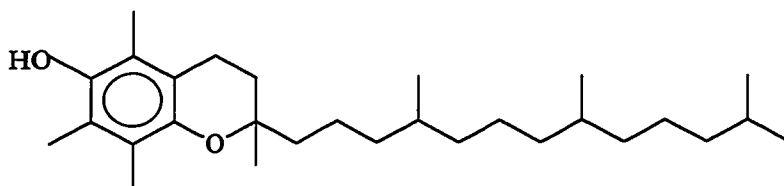


Figure 2.2 Structure of α -tocopherol (adapted from Halliwell & Gutteridge, 1999)

β -carotene

β -carotene is a major carotenoid precursor of vitamin A. β -carotene is also present in cellular membranes. Carotenoids are C_{40} isoprenoids or tetraterpenes which are found in all green tissues and are located only in the chloroplast. These pigments have an effective mechanism of scavenging ROS and preventing the generation of these species (Pallett & Young, 1993). However, the role of β -carotene as an antioxidant in humans is not clear. A recent study of plasma β -carotene concentrations and a randomised, controlled clinical trial of supplementation showed that persons with high initial plasma levels ($>0.52 \mu\text{mol/l}$) had a lower rate of death from all causes including cardiovascular diseases (Greenberg *et al.*, 1996). However supplementation of subjects with low initial concentrations for 3 years did not lower mortality (Greenberg *et al.*, 1996). Furthermore while epidemiological evidence supports a protective role for β -carotene against cancer (Torun *et al.*, 1995), supplementation of lung cancer patients with β -carotene showed no benefit and may have had an adverse effect (Omenn *et al.*, 1996). The result may be explained by the lower antioxidant efficiency of β -carotene at high oxygen tension. The formation of carotenoid-

peroxyl radical under high oxygen tension which is less stable than β -carotene adduct-radical may promote autoxidation of β -carotene or PUFAs (Everett *et al.*, 1996; Rice-Evans *et al.*, 1997). The α -tocopherol and β -carotene (ATBC) Cancer Prevention Study implemented in Finland during the 1980s (Albanes *et al.*, 1996) was a placebo-controlled, randomized study to test the hypothesis that α -tocopherol and β -carotene supplements prevent lung and other cancers. Results showed that the group with vitamin E supplements had lower incidence of cancers of the prostate and colorectum but higher stomach cancer than those without supplement. No advantage was found for the group receiving β -carotene in terms of cancer incidence but rather an increase of lung, prostate and stomach cancer cases was reported. A lower rate of lung cancer was observed for those with initially higher serum and dietary levels of both α -tocopherol and β -carotene levels (Albanes *et al.*, 1995). The biological function of β -carotene in humans, therefore, remains a challenging question owing to conflicting evidence between epidemiological and interventional studies.

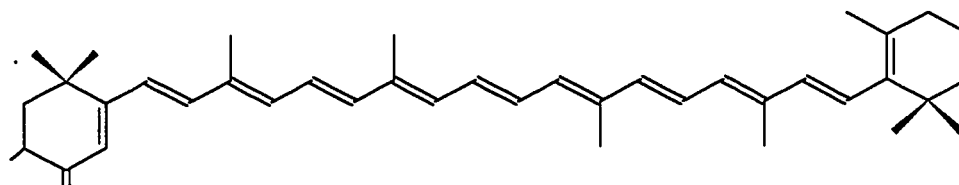


Figure 2.3 Structure of β -carotene (adapted from Halliwell & Gutteridge, 1999)

Vitamin C

Vitamin C is a group of compounds which possess the biological activity of ascorbic acid ($pK_1=4.17$, $pK_2=11.57$), including L-ascorbic acid, dehydro-L-ascorbic acid, D-isoascorbic acid and dehydro-D-isoascorbic acid (Macrae *et al.*, 1993). The biological functions of vitamin C include prevention of scurvy, acceleration of hydroxylation reactions in collagen, carnitine and norepinephrine synthesis (Bode, 1997; Benzie, 1999). Vitamin C is found in both plants and animals, occurring as ascorbic acid and dehydroascorbic acid. The stability of vitamin C is low and the level in food decreases dramatically during storage and cooking (Combs, 1992). Vitamin C acts on many biological systems, including immune function, neuroendocrine peptides, carnitine synthesis, biosynthesis of collagen and iron absorption (Jacques, 1992). Besides its non-antioxidant role, vitamin C can react with and scavenge many kinds of free radicals such as singlet oxygen, superoxide, hydroxyl radicals, and can react directly with aqueous, chain-carrying peroxy radical (Bendich *et al.*, 1986). It is thought that vitamin C can also regenerate the reduced form of α -tocopherol and urate (Maples & Mason, 1988; Padh, 1991). Since ascorbic acid cannot be synthesised by humans, dietary intake is the only way to meet requirements. Nutrient reference range or RDA of vitamin C ranges from 40 mg to 60 mg respectively. Levine and co-workers, however, suggested the RDA should be increased to 200 mg daily. This figure was based on a pharmacokinetic study of vitamin C (Levine *et al.*, 1996). Megadoses of vitamin C may affect cholesterol metabolism and so lower plasma cholesterol (Ginter, 1989) though some of the reported effects may owing to interference in cholesterol measurement (Benzie & Strain, 1995). Human fasting plasma ascorbate concentration ranges from 45 to 90 $\mu\text{mol/L}$ (Gutteridge &

Halliwell, 1994; Benzie *et al.*, 1998), but intracellular levels can reach millimolar concentrations (Washko *et al.*, 1993). Plasma vitamin C levels correlate negatively with the prevalence of cardiovascular disease and cancer (Benzie, 1998; Gey, 1998).

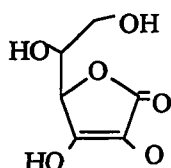


Figure 2.4 Structure of ascorbic acid (adapted from Halliwell & Gutteridge, 1999)

Glutathione

The antioxidant function of GSH is achieved by oxidation of the sulfhydryl (SH) group of cysteine: reaction with a second oxidised GSH molecule forms oxidised glutathione (GSSG). GSSG can be recycled by glutathione reductase at the expense of NADPH (Bunker, 1992). Besides being a substrate for glutathione peroxidase and for dehydroascorbate reductase, glutathione is a scavenger of hydroxyl radicals and singlet oxygen. Glutathione is present mainly intracellularly and is rich in liver tissue with about 4 $\mu\text{mol/g}$ (Halliwell & Gutteridge, 1989).

Uric acid

Uric acid (pKa 5.4) is the end product of purine metabolism in humans and circulates in plasma as sodium urate at concentrations of 150–450 $\mu\text{mol/L}$. Uric acid is derived from nucleic acid breakdown and is also synthesised from simple compounds in the body (Kaplan *et al.*, 1995). Uric acid may be an important endogenous antioxidant helping to replace ascorbic acid and is thought to have increased in level during primate evolution (Ames *et al.*, 1981; Ames, 1988; Cutler, 1991). Uric acid may be involved in scavenging oxygen radicals in the aqueous phase and may act as a chain breaking antioxidant (Niki *et al.*, 1985). Davies and co-workers have demonstrated that an important antioxidant property of uric acid is its ability to form stable co-ordination complexes with iron ions, inhibiting Fe^{3+} -catalysed ascorbate oxidation (Davies *et al.*, 1986). However, the importance of uric acid as a physiological antioxidant is not clear. Uric acid is reported to be an independent risk factor for CHD (Ebenbichler *et al.*, 1995), and levels of uric acid have been reported to correlate directly with an index of oxidative stress (Benzie & Strain, 1996a). Superoxide and hydrogen peroxide were found to be secreted by polymorphonuclear leukocyte after the stimulation by uric acid may contribute to the pro-oxidant activity of uric acid (Thomas, 1992).

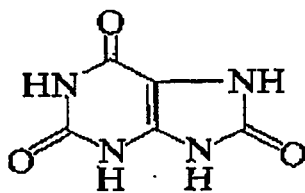


Figure 2.5 Structure of uric acid (adapted from Halliwell & Gutteridge, 1999)

Bilirubin

Bilirubin is generally regarded as a potentially cytotoxic waste product derived from the breakdown of haemoglobin when red blood cells are destroyed (Kaplan *et al.*, 1995). The concentration of bilirubin in plasma of normal adult is about 5 to 17 $\mu\text{mol/L}$ (Bhagavan, 1991). Bilirubin can scavenge peroxy radicals *in vitro*. The antioxidant activity of bilirubin in liposomes under 2% oxygen was found to be higher than that of α -tocopherol, and bilirubin may have a role as a chain-breaking antioxidant (Stocker *et al.*, 1987). Bilirubin was also suggested to be an efficient co-antioxidant for α -tocopherol (Neuzil & Stocker, 1994), and bilirubin may have important antioxidant function in certain situations, e.g. in the gut and during the neonatal period, where levels are high normally (Benzie, 1996a).

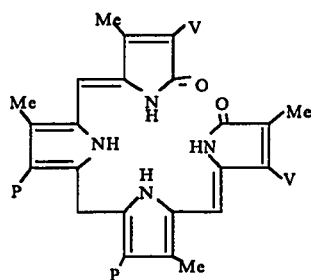


Figure 2.6 Structure of bilirubin (adapted from Halliwell & Gutteridge, 1999)

Ubiquinol/ubiquinone

Ubiquinol/ubiquinone are redox components of the mitochondrial electron-transport system, but the dynamics of antioxidant function are not clear. Ubiquinol may act as an antioxidant by donating its hydrogen to the prooxidant, for example $\text{O}_2^{\bullet-}$. Ubiquinol is present in human low-density lipoprotein (LDL) but the plasma concentration is very low and only about half of LDL particles

contain ubiquinol (Stocker & Bowry, 1996). α -TOH can act as either an antioxidant or pro-oxidant for lipid in lipoprotein and the presence of $\text{CoQ}_{10}\text{H}_2$ was reported to suppress the pro-oxidant activity of α -tocopherol (Thomas *et al.*, 1997). Ubiquinone may therefore have the ability to maintain an efficient recycling of TO^\bullet (Kagan *et al.*, 1996).

Ubiquinol 10 is the most abundant form of ubiquinol in the diet. Dietary supplementation with ubiquinol 10 was investigated by Mohr *et al.*. They showed that ubiquinol 10/ubiquinone 10 ratio was unaffected by oral supplementation. Supplementation with ubiquinone 10 will increase ubiquinol 10 in plasma so that resistance of LDL to oxidation can be increased (Mohr *et al.*, 1992).

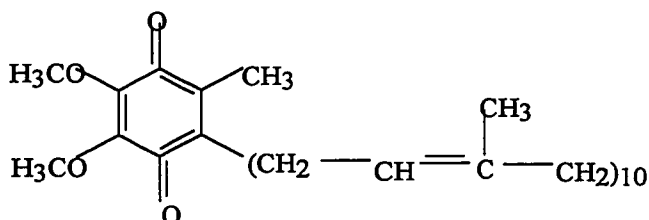


Figure 2.7 Structure of ubiquinone (adapted from Cadenas & Packer, 1992)

Flavonoids

Flavonoids are polyphenolic compounds that occur in foods of plant origin (Cook & Samman, 1996). Quercetin is the major compound of the flavonol subclass of flavonoids. Flavonoids reportedly scavenge oxygen radicals such as superoxide anion and even non-radical singlet oxygen and prevent oxidation of LDL *in vitro* (Bors *et al.*, 1996). The inhibitory action of quercetin on lipid peroxidation of lecithin liposomes and in rat liver microsomes was demonstrated (Afanas'ev *et al.*, 1989). This action is explained by both metal chelating and

antioxidative properties of flavonoids. The antioxidant activity is the effect of the hydrogen-donating capacity of the phenolic groups (Rice-Evans *et al.*, 1995). An inverse relationship between coronary heart disease mortality and intake of quercetin was observed in Dutch elderly men (Hollman *et al.*, 1996), but data linking flavonol intake and CHD are conflicting and further study is needed in this important area (Hertog *et al.*, 1997).

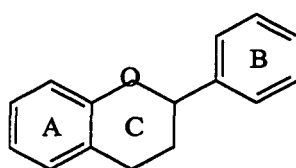


Figure 2.8 Generic Structure of flavonoids (adapted from Cook & Samman, 1996)

Table 2.5 Major classes of flavonoids and their sources (adapted from Cook & Samman, 1996; Rice-Evans *et al.*, 1996)

Flavonoids	Dietary Sources
Flavonols	Teas and red wine
Flavones	Fruits skin, celery and parsley
Flavanones	Citrus fruits and their peel
Flavanols	Teas
Anthocyanidins	Red grape , red wine, cherry, raspberry, strawberry, coloured fruit and peel
Isoflavones	Soybeans

A study by Hertog *et al.* showed the major flavonoid in the European diet was quercetin. The second important species was kaempferol, followed by myricetin, luteolin and apigenin. The dietary sources of flavonoids include tea, onions and apples (Hertog *et al.*, 1993), however the extent to which dietary

flavonoids are absorbed and whether or not short- and long term ingestion of these can significantly enhance antioxidant defence is not yet known.

α -Lipoic Acid

α -Lipoic acid is an antioxidant which is not soluble in water. α -lipoic acid is considered as an antioxidant since a number of experimental studies showed its ability to scavenge hydroxyl radicals, hypochlorous acid and singlet oxygen. α -lipoic can be synthesised in the body and is also taken in the diet (Packer *et al.*, 1996). Both the reduced and oxidised forms of α -lipoic acid may act as an antioxidant. α -lipoic acid, the oxidised form, does not scavenge superoxide radical, peroxy radicals and hydrogen peroxide but may chelate transition metals. However, the reduced form of α -lipoic acid, dihydrolipolic acid (DHLA), can scavenge superoxide radical and peroxy radical (Packer *et al.*, 1996). Plasma concentration of α -lipoic acid is 8-200 nmol/l and is 260-1160 nmol/l for DHLA (Teichert & Preiss, 1992). Epidemiological studies have suggested a role for this antioxidant in the prevention of some oxidant-related diseases. As α -lipoic acid is readily absorbed from the diet, it is a potential antioxidant for therapeutic use (Packer *et al.*, 1996). However, it should be noted that the very low levels mean that most lipoprotein do not contain any α -lipoic acid or DHLA.



Figure 2.9 Structure of α -lipoic Acid (adapted from Halliwell & Gutteridge, 1999)

Active-site Component of Enzymes (Trace Metals)

Catalytic sites of antioxidant enzymes contain certain essential metal ions for their proper function (Table 2.6). Copper, zinc, manganese and iron are present in the active site of different Superoxide Dismutases (SODs) (Halliwell & Gutteridge, 1989). Another important enzyme, glutathione peroxidase (GSH-Px) contains selenium in its structure (Rotruck *et al.*, 1973). These trace metals are sometimes referred to as 'antioxidants': however, while a deficiency of these may result in a lack of enzyme activity, enzyme activity is not usually increased when levels are raised above sufficiency, and excess copper, iron, manganese is not desirable since transition metals can catalyse oxidation by changing their valency state.

Table 2.6 Summary of active-site component of enzymes

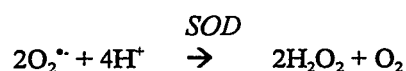
Copper	active-site catalyst of Cu,Zn- superoxide dismutase
Zinc	active-site catalyst of Cu,Zn- superoxide dismutase
Manganese	active-site catalyst of mitochondrial superoxide dismutase
Selenium	active-site catalyst of glutathione peroxidases

Antioxidant Enzymes or Proteins

Antioxidant enzymes interact in a co-ordinated manner with each other to remove harmful reactive species, and a balance in the activities of these enzymes should be maintained. Some proteins can bind to transition metals so that the free metal concentration can be minimised in order to reduce the formation of reactive species initiated by transition metal (Yu, 1994).

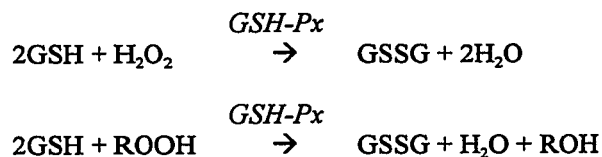
Superoxide Dismutase (SOD)

The best-known antioxidant enzyme is SOD. There are several different SODs but all catalyse the dismutation of $O_2^{\cdot -}$ to hydrogen peroxide (H_2O_2). This is an important physiological mechanism in aerobic organisms. Almost all aerobic eukaryotes contain SOD. Cu, Zn-superoxide dismutase (Cu, Zn-SOD) is found in the cytosol, nucleus, and some in peroxisomes. A Mn-superoxide dismutase (Mn-SOD) is found in mitochondria, and sometimes in the cytosol (Gutteridge & Halliwell, 1994). The reaction of SOD is shown below:



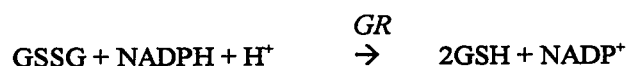
Glutathione Peroxidase (GSH-Px)

Glutathione peroxidase catalyses the removal of H_2O_2 and other hydroperoxides with the consumption of reduced glutathione (Yu, 1994). The rate of removal of H_2O_2 by this enzyme is high (Gutteridge & Halliwell, 1994). Activity of GSH-Px is high in liver tissue and moderate in heart, lung and brain (Halliwell & Gutteridge, 1989).



Glutathione Reductase (GR)

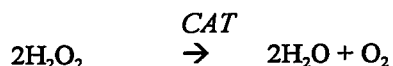
Glutathione reductase catalyses the NADPH-dependent reduction of oxidised glutathione, thus restoring supplies of reduced glutathione.



Glutathione reductase is a flavoprotein and is found in the cytosol and mitochondrial matrix. Its activity is also associated with other membrane-bound organelles such as the endoplasmic reticulum (Halliwell & Gutteridge, 1989).

Catalase (CAT)

Catalase catalyses reduction of H_2O_2 to water. Catalase is a large enzyme that contains haem-bound iron at the active site. Catalase is found in peroxisomes. The affinity for H_2O_2 is low, and so it requires a high H_2O_2 concentration to work fast, but its capacity to destroy H_2O_2 is very large (Gutteridge & Halliwell, 1994).



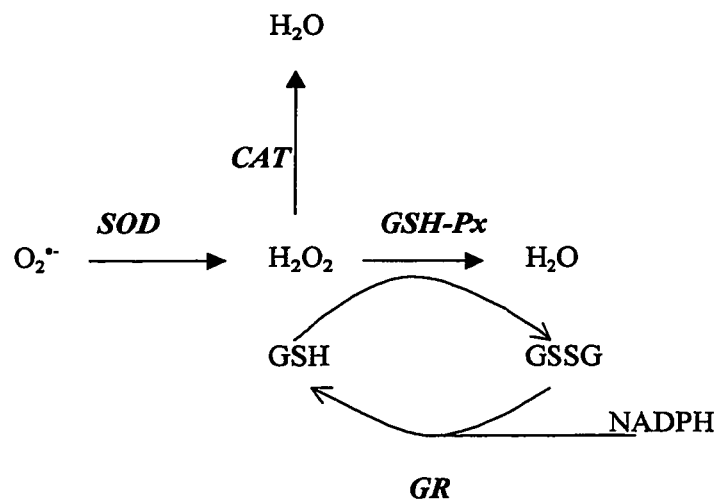


Figure 2.10 Co-operation between antioxidant enzymes

Antioxidant Proteins: Caeruloplasmin, Ferritin and Transferrin

Ferritin and transferrin bind iron in plasma and the extracellular compartment. Transition metals are involved in metal-catalysed oxidations and reactions leading to hydroxyl radical production from superoxide and peroxides, so these non-enzyme proteins contribute to the antioxidant protection of the body (Davies, 1994). The ferrioxidase activity of caeruloplasmin converts Fe^{2+} to Fe^{3+} for incorporation into ferritin, but without concomitant production of $\text{O}_2^{\cdot-}$. Ferric ion is unreactive to H_2O_2 (Cadenas, 1995; Stocks *et al.*, 1974). Transferrin and ferritin can minimise the occurrence of free iron in plasma or inside cells respectively and are relatively 'safe' storage form of iron (Cadenas, 1995). In iron overload situation such as repeated transfusion and idiopathic haemochromatosis, or if copper or iron is released from binding, the Fenton reaction may occur. This is shown below:

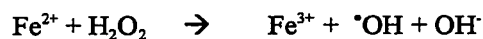


Table 2.7 Summary of functions or properties of antioxidant enzymes or proteins

Name	Role/Reaction catalysed	Site
Superoxide Dismutase (SOD)	$2\text{O}_2^{\cdot -} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$	cytosol, nucleus, peroxisomes, mitochondrial
Glutathione Peroxidase (GSH-Px)	$2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}$ $2\text{GSH} + \text{ROOH} \rightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O}$	cytosol, mitochondrial matrix
Glutathione Reductase (GR)	$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+$	cytosol, mitochondrial matrix, some membrane-bound organelles
Catalase (CAT)	$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$	peroxisomes of endoplasmic reticulum, mitochondria microsomes and cytosol
Caeruloplasmin	Transition-metal storage (copper), catalyses the 'safe' conversion of Fe^{2+} to Fe^{3+} which is unreactive towards H_2O_2	Plasma
Ferritin	Transition-metal storage (conversion of Fe^{2+} to Fe^{3+})	Cytosol
Transferrin	Transition-metal storage (Fe^{3+})	Plasma

In summary, those antioxidant enzymes and proteins such as SOD, GSH-Px, GR, CAT, caeruloplasmin, ferritin and transferrin are essential for preventing the formation of reactive species. And the small molecules such as ascorbic acid, α -tocopherol and uric acid are responsible for scavenging those reactive species which are formed. Antioxidants work in a co-ordinated manner and form the 'defence system' of the body to resist the challenge of aerobic life.

Pro-oxidant Effect of Antioxidants

Although antioxidants play an important role in opposing oxidative stress, antioxidants in certain circumstances can exert pro-oxidant effects. Ascorbic acid at greater than 1 mmol/l concentration shows cytotoxicity to leukemic cells and the toxic effect was thought to be due to ascorbyl radical (Sakagami *et al.*, 1997). Plant phenolic compounds at high concentrations demonstrate pro-oxidant effects when they interact with iron ions (Scott *et al.*, 1993). Carnosol and carnosic acid found in the herb rosemary stimulate DNA damage in the bleomycin assay but can scavenge $^{\bullet}\text{OH}$ in the deoxyribose assay (Aruoma, *et al.*, 1992). Phenolics forming complexes with Fe^{3+} show pro-oxidant effects because the Fenton reaction can still proceed (Puppo, 1992), i.e. the bound transition metal is still redox active. Therefore the concentration or amount of certain substances is critical for defining their roles. A very high concentration of an 'antioxidant' may become a pro-oxidant. Hence the balance of defence mechanisms is not only related to the relative amount of reactive species and antioxidants, but also the absolute amount of antioxidants.

Diseases Associated With Increased Oxidative Stress

We know that reactive species can cause damage to various kinds of biomolecules, affecting their structure and functional properties. The body may not function properly if the damage is not repaired in time. If there is an imbalance of antioxidants and pro-oxidants therefore, especially if this is sustained for long enough, oxidative stress may make a significant contribution to risk, development and severity or progression of disease. Many diseases are thought to be related to

increased oxidative stress (Ames *et al.*, 1993). These include cardiovascular diseases, autoimmune diseases, Parkinson's disease, ischaemia and reperfusion injury, cancer, cystic fibrosis and diabetic complications (Halliwell & Gutteridge, 1989; Diplock, 1994).

Skin Pathologies

Solar ultraviolet light exposure is associated with many skin pathologies, including melanoma and non-melanoma skin cancer, photoaging and photosensitisation (Packer, 1994; Cooke *et al.*, 1996). The free radical hypothesis of UV-induced damage provides an explanation for the rationale of these diseases (Packer, 1994). The action of UV light forms ROS in skin. Although antioxidant defences in skin, such as α -tocopherol and ubiquinol, eliminate ROS, these defences will be overwhelmed if the amount of UV light is great enough, and eventually will cause damage to lipids, DNA and proteins (Packer, 1994). The protein molecules may be cross-linked and elasticity is lost. DNA damage will increase the risk of mutagenesis or carcinogenesis.

Cancers

Oxidative damage to DNA and protein can result the alteration of the interaction between DNA and protein, leading to mutagenesis and carcinogenesis (Halliwell & Gutteridge, 1989; Shigenaga & Ames, 1993). Lack of antioxidants to counteract these adverse effects may be a cause of cancer. Many studies have been carried out to investigate the relationship between antioxidant status and cancer risk (Stahelin *et al.*, 1991). Results of several studies on cancer mortality were summarised and showed that poor plasma antioxidant status was associated with increased cancer risk (Gey, 1991; Gey, 1993; Gey, 1998). Lower levels of

carotene, vitamins A, C and E were associated with increased risk of cancer of lung, gastrointestinal, skin melanoma, stomach, prostate, colorectal, pancreas, urinary systems, breast and female reproductive organs (Gey, 1993; Benzie, 1998; Gey, 1998).

Atherosclerosis and Cardiovascular Disease

Atherosclerosis is a disease of arteries that is characterised by a local thickening of the innermost part of the vessel. Current opinion suggests oxidised low density lipoprotein (LDL) is the primary atherogenic form of the lipoprotein, rather than native LDL (Steinberg *et al.*, 1989; de Graff *et al.*, 1991; Kendler, 1995; Singh & Downing, 1995). Transition-metal ions (Cu^{2+} or Fe^{2+}) are believed to react with lipid hydroperoxide (LOOH) contained in LDL to form peroxyradicals (LOO^{\bullet}) and alkoxy radicals (LO^{\bullet}). $^{\bullet}\text{OH}$ radical formed from Haber-Weiss or Fenton reaction initiate lipid peroxidation by abstracting a hydrogen atom from an unsaturated fatty acid, leading to the formation of a carbon centred radical. The carbon radical is likely to react with O_2 and forms lipid hydroperoxide (Benzie, 1996a). LOO^{\bullet} and LO^{\bullet} radicals can propagate the lipid peroxidation reaction in polyunsaturated fatty acid of LDL particles. LDL containing oxidised lipids is formed and lecithin will be converted to lysolecithin. Degradation of apoprotein B and the conjugation of fragments of oxidised fatty acids with apoprotein B will then follow. Since the change of the surface of apoprotein B can be recognised by the macrophage scavenger receptor, the macrophage will take up the oxidised LDL (Steinberg *et al.*, 1989). The uptake of oxidised LDL contributes to the accumulation of cholesteryl esters in foam cells of the budding atherosclerotic lesion. The susceptibility for oxidative modification

of LDL and the plasma LDL-cholesterol concentration correlate to the severity of coronary atherosclerosis. Also, susceptibility of LDL to oxidation is related to the triglyceride composition of the LDL fraction. Small dense LDL, and LDL with higher concentration of PUFA may be more easily oxidised (de Graff *et al.*, 1991). The oxidation hypothesis suggests that LDL oxidation accelerates atherogenesis (Thomas, 1995), indicating that lipid peroxidation enhances atherosclerosis, leading to coronary artery disease (Fruchart & Duriez, 1994).

Diabetes Mellitus and Its Complications

Autoimmune phenomena play an important role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM), and there is a strong genetic link. However, environmental factors appear to affect the triggering of the genetic trait in IDDM, and this is true also for non insulin-dependent diabetes mellitus (NIDDM) (Virtanen & Aro, 1994). Superoxide radical production was reported to be increased in insulin-dependent diabetic patients (Nath *et al.*, 1984). Glycation and lipid peroxidation are thought to relate to increased oxidative stress and to contribute to the development of complications such as retinopathy, neuropathy and nephropathy. Plasma ascorbate levels are low in diabetic subjects, and supplementation of vitamins C and E reportedly showed beneficial effects in reducing glycation and lipid peroxidation (Vinson *et al.*, 1994). Low serum ascorbic acid level was identified in patients with diabetic macroangiopathy (Nagano *et al.*, 1996) and glucose tolerance improved with vitamin E supplementation (Paolisso *et al.*, 1993). There may, therefore, be a role for dietary antioxidant therapy in slowing the development or progression of diabetic complications.

Parkinson's Disease

The pathology of neurodegenerative diseases may involve oxidative damage to the nervous system (Halliwell & Gutteridge, 1989). Current opinion suggests that toxic agents endogenously produced or from the environment damage the nigral cells in Parkinson's disease. The decrease in the level of reduced glutathione in the substantia nigra in Parkinson's disease may be the result of neuronal loss, since positive correlation was found between neuronal loss and decreased glutathione level. The decrease in reduced glutathione also lowers the ability of the tissue to detoxify H_2O_2 (Ebadi, 1996). The activity of mitochondrial SOD, zinc content and lipid peroxidation are increased, which also indicates increased oxidative stress in substantia nigra in this condition (Jenner, 1992). Current treatment strategies focus on lowering oxidative stress or increasing resistance to it. High-dose vitamin E and vitamin C were given to patients in order to slow progression of Parkinson's disease. However to date there is no clinical evidence to show the effectiveness of this approach (Ahlskog, 1990).

Pancreatitis

Preliminary indirect observations suggest oxygen free radicals are produced and add to the tissue damage sustained in acute, acute recurrent and chronic pancreatitis (Schoenberg *et al.*, 1995). Oxygen radicals may cause lipid peroxidation leading to pancreatic cell death, or may attract neutrophils or plugging of the vessels of pancreas tissue. Whether ROS are the cause or effect of pancreatitis remains unclear, and many hypotheses have been put forward (Schoenberg *et al.*, 1995). Oral administration of vitamins C, E, β -carotene and selenium could reduce the number or severity of attacks and is a possible

alternative to surgery (Scott, 1997). However, the utility of this approach has yet to be established.

Ischaemia and Reperfusion Injury

The hypothesis that ROS are produced during reperfusion of ischaemic tissue was first proposed in 1981 by Granger and co-workers (Granger *et al.*, 1981). Post-ischaemic reperfusion may lead to a pattern of damage, and the damage is believed to occur during the reperfusion period rather than ischaemic period (Ferrari, 1994). Reperfusion-induced microvascular injury is thought to be owing to xanthine oxidase-derived oxidants produced after reoxygenation of ischaemic tissue (Chen *et al.*, 1995). The enzyme xanthine dehydrogenase (XDH) is converted to xanthine oxidase (XO) during ischaemia, and ATP is degraded to hypoxanthine which will accumulate in ischaemic tissue. When the supply of molecular oxygen is restored during reperfusion, XO will use hypoxanthine and oxygen as substrates and $O_2^{\cdot -}$ is formed (Ferrari, 1994).

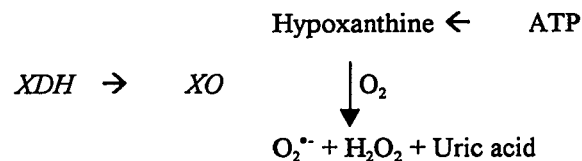


Figure 2.11 Production of superoxide radical during ischaemia

In summary, since formation of reactive species is involved in a number of diseases. ROS either are part of the cause or add to further damage of tissues or both. The prevention or at least delaying the onset of disease may be achieved by enhancing the defence system against reactive species. Dietary modification with

antioxidant rich foods or supplementation with antioxidants are the possible strategies to fight oxidative stress related diseases.

Other oxidative stress-related diseases

Cataracts and rheumatoid arthritis are also the disorders associated with increased oxidant stress (Gambhir *et al.*, 1997; Araujo *et al.*, 1998; Strain & Benzie, 1998a). Diminished antioxidant capacity in those patients was reported (Mares-Perlman *et al.*, 1995; Mazzetti *et al.*, 1996; Comostock *et al.*, 1997). Prolonged exercise, although not a disease, can also lead to a lower antioxidant capacity (Venditti *et al.*, 1996). Optimising antioxidant micronutrients intake may delay or avoid these diseases or help the body cope in situations of increased stress (Muggli, 1993).

Potential Role Of Dietary Agents In Lowering Risk Of Chronic Diseases Associated With Increased Oxidative Stress

Antioxidants and Nutrition

Although it is unlikely that ROS are the cause of all cancers, observations imply ROS promote carcinogenesis and increase the risk of several types of cancer (Machlin, 1995). Epidemiological findings show high consumption of vitamin C-containing foods is associated with a reduction in risk of all cancers (Gey, 1993; Benzie, 1998). The effect may be due to the inhibition of nitrosoamine formation during food processing; nitrosoamines are procarcinogens in food. High intake of carotene-rich fruits and vegetables is also associated with lower cancer risk (Machlin, 1995; World Cancer Research Fund, 1997). But supplementation with β -carotene was reported to be associated with an increase in

lung cancer incidence, and death by cardiovascular disease (Omenn *et al.*, 1996). A large cross-cultural epidemiological study by Gey *et al.* demonstrated the strong inverse relationship between heart disease mortality and plasma vitamin E level. This was probably due to different dietary intakes of fruit and vegetables in the study populations (Gey *et al.*, 1991). Also, dietary supplementation of antioxidants vitamins C and E reportedly lowered the risk of cataracts (Muggli, 1993).

Since increased oxidative stress may increase risk of disease, lowering oxidative stress through dietary strategies aimed at increasing antioxidant defence is a possible way to lower the risk of these oxidative stress related diseases. Results of dietary antioxidant treatment to date have not been shown to be very effective, however, and epidemiological and intervention studies have shown inconsistent results (Ambrosone *et al.*, 1994; Gutteridge & Halliwell, 1994; Vinson *et al.*, 1994; Greenberg *et al.*, 1996). Nevertheless, the clear relationship between diet and disease on a population basis, in association with experimental data, provide a clear biochemical rationale for health benefits of increasing antioxidants in the diet.

Recent studies on the dietary intake or plasma concentration of vitamin C showed a strong inverse relationship to subsequent risk of death from stroke but not from coronary heart disease (Gale *et al.*, 1995). A comprehensive review by Gey summarised the antioxidant nutrients of plasma levels and the related risks to cardiovascular diseases and potential protective plasma vitamin levels. These level were suggested to be $> 50 \mu\text{mol/L}$ for vitamin C and vitamin C/vitamin E ratio $>$

1.3-1.5, ≥ 5.0 $\mu\text{mol}/\text{mmol}$ for lipid-standardised vitamin E (α -tocopherol/cholesterol) and > 0.4 $\mu\text{mol}/\text{L}$ for β -carotene. Gey concluded that suboptimal levels of each factor increase the risk singly, or increases multiplicatively if in combination. These antioxidant factors could be stronger predictors of coronary heart disease than classical risk factors such as cholesterol and blood pressures levels (Gey, 1995; Gey, 1998).

Many constituents of food contain natural antioxidants, enzymatic and non-enzymatic. Non-enzymatic antioxidants such as Vitamins C, E and β -carotene may still be active after heat treatment but enzymatic antioxidants are normally denatured in food processing. In addition, some antioxidants are known to form during heat-processing and microbial fermentation of foods. These include reductones, melaniodins, pyrroles and pyridines, which are components of amino-carbonyl reaction (Maillard reaction) products (Eriksson & Na, 1993; Namiki, 1988). Reactants involved in the Maillard reaction are amino acids, peptides, proteins, amines, ammonia, reducing sugar and carbonyl compounds. Volatile compounds formed from heated foods and nitrosylmyoglobin also process antioxidant activities. Sometimes food may be enriched with antioxidants since commercial antioxidants are added for preservation or nutritional enhancement (Eriksson & Na, 1993). Intake of food enriched with antioxidants may improve antioxidant defence if these antioxidants are absorbed. As a result of increased antioxidant intake, the damage caused by reactive species could be lowered and hence there would be a decrease of risk of free radical-related diseases. Therefore, knowing the antioxidant level, stability and bioavailability of dietary constituents

could help formulate useful dietary strategies to achieve and maintain a good antioxidant status through intake of an antioxidant rich diet.

Exogenous Sources of Antioxidants: tea, wine, fruits, vegetables, herbs and drugs and their putative beneficial affects

Tea

Tea (*Camellia sinensis*) has been consumed for several thousand years as a favoured beverage in China and is now consumed throughout the world. Green tea is produced by steaming and heating the rolled leaves soon after chopping (Graham, 1992). Green tea is consumed mainly in Asia and parts of North Africa and comprises about 20% of world production. Oolong tea, which is an intermediate between green and black tea, comprises about 2% of global consumption. The remaining global consumption is in the form of black tea (Goldbohm *et al.*, 1996), which is produced by allowing the leaves to wither for about 6 hours after chopping and rolling (Weisburger, 1996). Both green and black tea contain various polyphenolic compounds which possess antioxidant activities. Flavonoids (mainly catechins) including (-)-epigallocatechin gallate, (-)-epicatechin gallate, (-)-epigallocatechin, (-)-epicatechin, (+)-catechin and gallic acid can be found in green tea. The amount of most of these substances in black tea is significantly lower (Balentine, 1992). Theaflavins and thearubigins, which are the polymerised oxidation products of the catechins and which also have marked antioxidant effects, are produced in the process of black tea manufacturing (Balentine, 1992; Sano *et al.*, 1995).

Table 2.8 Polyphenols found in tea leaves (*Camellia sinensis*) (adapted from Balentine, 1992)

Component	Amount (% in dry extract)	Amount (% in dry extract)
	Green tea	Black tea
Catechins	34.0	4.2
Theaflavin	-	1.8
Thearubigin	-	17.0
Flavonols	0.4	-

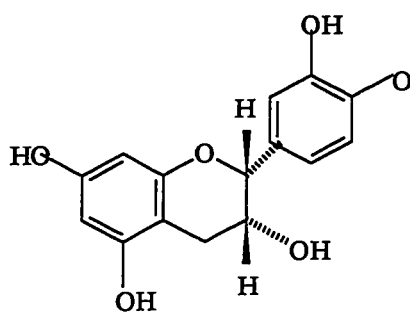


Figure 2.12 Structure of epicatechin (EC) (adapted from Graham, 1992)

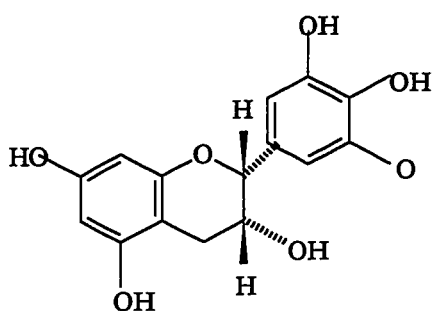


Figure 2.13 Structure of epigallocatechin (EGC) (adapted from Graham, 1992)

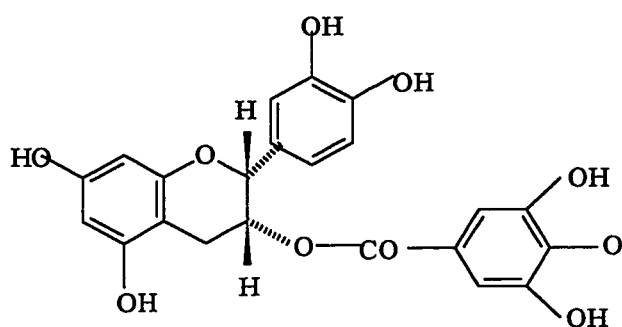


Figure 2.14 Structure of epicatechin gallate (ECG) (adapted from Graham, 1992)

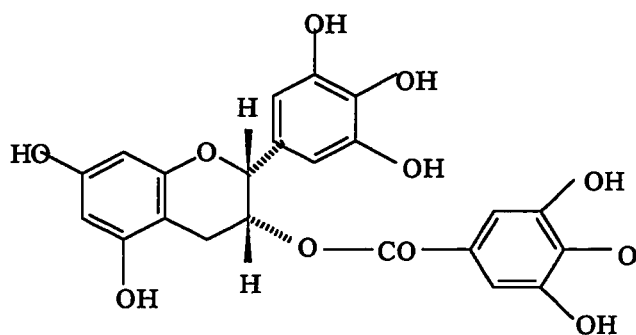


Figure 2.15 Structure of epigallocatechin gallate (EGCG) (adapted from Graham, 1992)

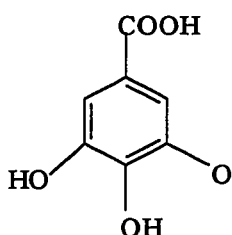


Figure 2.16 Structure of gallic acid (adapted from Cook & Samman, 1996)

Retrospective and prospective cohort studies have shown an inverse relationship between intake of green tea and gastric cancer, cardiovascular diseases and indicators of liver dysfunction (Yu *et al.*, 1995; Imai & Nakachi, 1995). Polyphenols from tea have been reported to suppress the oxidative modification of LDL *in vitro*. (Miura *et al.*, 1994). Green and black tea demonstrated an inhibitory effect on tissue lipid peroxidation *ex vivo* in rat liver and kidney (Sano *et al.*, 1995). Green tea is also reported to block oxidative DNA damage to the liver and to lower hepatotoxicity in rats treated with 2-nitropropane (Hasegawa *et al.*, 1995). Green tea is also reported to have a hypocholesterolaemic effect (Yang & Koo, 1997). Not all studies report benefit,


however, and no significant effect on risk factors such as blood pressure, blood lipids and bowel habit was observed in association with black tea drinking (Bingham *et al.*, 1997). Results from the investigation by Goldbohm and co-workers do not support the hypothesis that consumption of black tea protects against colorectal, lung, stomach and breast cancers in humans (Goldbohm *et al.*, 1996). The Caerphilly study by Hertog *et al.* observed a weakly positive relationship between flavonol intake and ischaemic heart disease in Welsh men (Hertog *et al.*, 1997). Also, the absorption and metabolism of tea antioxidants is not clear (Das, 1979; Gugler *et al.*, 1975; Hollman *et al.*, 1996). These inconsistent and conflicting results may be owing to the differences in types or form of tea used in the different studies, differences in methodologies, differences in social class or smoking habit. Therefore, further exploration on the absorption and effect of tea is necessary before any conclusion can be drawn as to its health benefits in terms of its antioxidant potential.

Wine

Numerous studies on the effect of alcohol and wine consumption on overall mortality (Leger & Cochrane, 1979; Doll *et al.*, 1994; Gronbaek *et al.*, 1995), antioxidant capacity (Whitehead *et al.*, 1995), and on risk factors for atherosclerosis (Masarei *et al.*, 1986; Frankel *et al.*, 1993; Sharpe *et al.*, 1995) have been performed. *In vitro* studies with phenolic substances in red wine and normal human LDL showed that red wine has potent antioxidant properties toward oxidation of LDL (Frankel *et al.*, 1993). Also antioxidant levels in certain kinds of wine were determined in order to understand *in vitro* antioxidant power (Kanner *et al.*, 1994; Campos and Lissi, 1996). Day and Stansbie suggested the

acute production of urate after intake of alcohol may be the explanation for the associated post-ingestion increase of plasma antioxidant capacity (Day & Stansbie, 1995). However wine polyphenolics, if absorbed, could lower risk of oxidative stress-related disease in the same way as those in tea may. One obvious disadvantage of wine ingestion, however, is its ethanol content.

Table 2.9 Constituents of the total antioxidant activity of red wine (adapted from Miller & Rice-Evans, 1995)

Constituents	Contribution to total antioxidant activity
Catechin	Decreasing Contribution 
Gallic Acid	
Epicatechin	
Malvidin-3-glucoside	
Quercetin	
Myricetin	
Cyanidin	
Caffeic Acid	
Rutin	
Resveratrol	

Herbs

Estimation by the World Health Organisation indicates that up to 80% of the world population relies on traditional medicine (Taylor, 1996). Folk medicines of China usually include herbs in clinical practice. Ginseng is used because of the wide range of pharmacological actions it possesses. Recently, *in vitro* antioxidant activity of ginseng extract was reported and it showed the ability to inhibit

decomposition of fatty acids due to lipid peroxidation (Zhang *et al.*, 1996). Sheng Mai San (SMS) is a compound formulation in traditional Chinese medicine used for ischaemic heart disease. It comprises three species of herbs. Antioxidant activity was found mainly in one, *Schisandra chinensis*. *In vivo* protective effect of *Schisandra chinensis* extract on carbon tetrachloride-induced hepatotoxicity in mice, and scavenging superoxide radicals generated in animal model was demonstrated (Ko *et al.*, 1995; Ip *et al.*, 1996). A Japanese herb Sho-saiko-to-go-keishi-kashakuyaku-to (TJ-960) showed the ability to scavenge some types of free radicals *in vitro* and also to enhance the SOD level of hippocampus and hypothalamus in the aged rat. TJ-960 may be a potential agent against some neuronal symptoms (Hiramatsu *et al.*, 1992).

Extracts of rosemary and provencal herbs were reported to possess antioxidant activities, and were able to inhibit peroxidation of phospholipid liposomes. The main active ingredients in these herbal extracts are carnosol and carnosic acid (Aruoma *et al.*, 1992; Aruoma *et al.*, 1996).

Traditional medicines or herbs, therefore, may have significant antioxidant potential. The amount of such herbs taken, however, is generally low. The exception may be in traditional herbal remedies, such as ginger (*Zingiber officinale*) and lingzhi (*Ganoderma lucidum*), which are often taken regularly in fairly large amounts by our local population in Hong Kong.

Flavourings

Some of the flavourings reported to contain antioxidant activity include sesame and garlic. Experiments have demonstrated that sesame seed and its lignans can increase the vitamin E activity in rats even on low α -tocopherol diet (Yamashita *et al.*, 1992; Yamashita *et al.*, 1995). Garlic has been claimed to be effective in lowering blood lipid levels (Adler & Holub, 1997) and protecting against cancer in animal model (Fukushima *et al.*, 1997). *In vitro* antioxidant effects of garlic extract were identified (Phelps & Harris, 1993; Imai *et al.*, 1994) with allicin believed to be the active principle agent which was able to scavenge $\cdot\text{OH}$ (Prasad *et al.*, 1995).

Drugs

Halliwell and Gutteridge have suggested the possibility of non-steroidal anti-inflammatory drugs and other anti-inflammatory drugs that have antioxidant effects, since some of them can scavenge radicals such as $\cdot\text{OH}$ and HOCl . These drugs might also control oxidant reactions by changing the amount of oxidants produced by neutrophils, monocytes and macrophages (Halliwell & Gutteridge, 1989). Hence, investigation on the antioxidant power on various kinds of drug may lead to the discovery of additional therapeutic beneficial effects. Several drugs have been reported to have antioxidant activity including tamoxifen, carvedilol and captopril (Aruoma, 1995; Benzie & Tomlinson, 1998), however, whether this property could be a useful additional therapeutic effect is not yet clear and requires further study.

Measurement of Total Antioxidant Capacity of Biological Fluids

Antioxidant status in humans can be thought of as reflecting the equilibrium between antioxidants and pro-oxidants. Measurement of *in vitro* antioxidant power may be useful in assessing activity of drugs, foods etc., and in helping to assess the body antioxidant status. Different methods are available and these methods have different rationale and focus on different aspects of antioxidant status.

Total Radical Trapping Antioxidant Parameter (TRAP) and Its Various Forms

The original TRAP method employed an oxygen electrode to measure oxygen uptake in order to determine the delay in initiation of lipid peroxidation induced by decomposition of 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) (Wayner *et al.*, 1987; Mulholland & Strain, 1991). Recently, AAPH has been used to induce peroxidation and the loss of fluorescence of R-phycoerythrin is monitored (Ghiselli *et al.*, 1995). The lag-phase induced by plasma is compared with Trolox (a water soluble analogue of α -tocopherol) as standard. Protein interference can be eliminated by protein precipitation using ammonium sulphate (Ghiselli *et al.*, 1995; Serafini *et al.*, 1996). Since the assay is a one-by-one test, a very long time is required for analysis of a test series. This test is not suitable for very large numbers of specimens and fresh samples cannot be readily processed.

Oxygen Radical Absorbance Capacity (ORAC)

ORAC is a modification of the TRAP assay employing β -phycoerythrin, a light-harvesting protein, and 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxy radical generator. The radical generated will react with antioxidants present and the fluorescence from β -phycoerythrin will decrease due to the quenching effect. The area under the kinetic curve is proportional to the total ORAC of the sample, and results are obtained with reference to a standard solution of Trolox, a water-soluble analogue of α -tocopherol. It is claimed that oxidation reaction is driven to completion and does not depend on the initial concentration of the antioxidants so that problems associated with kinetics can be avoided (Cao *et al.*, 1993). A similar approach is applied in this assay as in the TRAP assay and the test can be automated with the COBAS FARA (Cao *et al.*, 1995). However, the reaction time is more than 1 hour, and plasma results are higher than with other methods, with greater than 90% of the ORAC reaction owing to presence of protein. Sensitivity, therefore, is poor for non-protein antioxidants.

2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) Assay

ABTS assay is also derived from the TRAP assay. This spectrophotometric method involves the ABTS radical cation generated from metmyoglobin. Antioxidants react with ABTS and this leads to a decrease of absorbance at 645, 734 and 815 nm. Trolox is used as the standard and the results are expressed as Trolox Equivalent Antioxidant Capacity (mmol/l TEAC) (Miller *et al.*, 1993; Miller & Rice-Evans, 1996). The assay is commercially available from Randox Laboratories Ltd (United Kingdom).

Ferric Reducing / Antioxidant Power Assay (FRAP)

This simple method is used to measure the ability of the sample to reduce Fe^{3+} to Fe^{2+} . Tripyridyltriazine is complex to Fe^{3+} at low pH. When the complex is reduced by the antioxidant, an intense blue colour develops and measurement of the absorbance can be made at 593 nm. In other words, this assay is measuring the electron donating or reducing power of antioxidants in the test sample rather than the ability to scavenge radicals generated in the sample. Automation with the COBAS FARA centrifugal analyser makes the assay fast, simple, inexpensive and the test shows high sensitivity (Benzie & Strain, 1996b; Benzie & Strain, 1999). Precision is higher than that of ORAC and Randox-TEAC methods (Cao & Prior, 1998). Slight modification of this assay can be used to measure ascorbic acid virtually simultaneously and specifically (Benzie, 1996b; Benzie & Strain, 1997a; Benzie & Strain, 1997b).

Assessing Oxidative Damage to Lipid and DNA

Determination of Lipid Peroxidation-derived Products

The determination of thiobarbituric acid reactive substances (TBARS) is a widely used method in studying oxidative stress-induced lipid peroxidation. The lipid peroxidation-derived product malondialdehyde (MDA) is a marker of lipid damage. The preexisting MDA and MDA formed from the decomposition of lipid hydroperoxides are measured. The degraded lipid peroxide end-products react with thiobarbituric acid (TBA) during heating in acid and a pink coloured adduct is formed. The absorbance or fluorescence intensity of the adduct is measured after organic solvent extraction. Since different versions of this test have been used comparison of results obtained in different laboratories is very difficult

(Coudray *et al.*, 1995; Benzie, 1996a). Although this assay is non-specific and problematic, it is still commonly used. Analysis of lipid hydroperoxide can also be done by reverse-phase high performance liquid chromatography (HPLC) with electrochemical detector. Separation of several cholesterol hydroperoxide species and phospholipid hydroperoxide species is achieved (Korytowski & Girotti, 1995), however, this is more demanding on equipment and technique. Measurement of low-density lipoprotein oxidation was first described by Esterbauer *et al.*. This group isolated the LDL by density gradient ultracentrifugation. Then the Cu^{2+} -catalysed oxidation was performed and the conjugated diene formation rate monitored at 234 nm (Esterbauer *et al.*, 1989). With the development of flow cytometry technique, response of the cell membrane to lipid peroxidation can now be detected by introducing the fluorescent dye followed by oxidant challenge (el-Rahman *et al.*, 1995; Makrigiorgos, 1997).

Measurement of DNA Damage

Oxidative stress can induce damage to DNA and such damage can contribute to the development of oxidative-stress related disease. A biomarker for DNA damage investigation is therefore necessary.

Gas chromatography-mass spectroscopy and high performance liquid chromatography with electrochemical detection can be used to measure 8-oxo-2'-deoxyguanosine, which is the oxidation product of DNA bases. However, it is susceptible to generation of artefacts during the lengthy extraction and processing of DNA prior to analysis (Collins *et al.*, 1996; Cooke *et al.*, 1996), and requires highly specialised equipment.

Single-cell gel electrophoresis assay (Comet Assay)

The comet assay is a sensitive technique which was first introduced in 1984 (Östling & Johanson, 1984) and was modified in 1988 to detect both single and double strand DNA breaks (Singh *et al.*, 1988). The comet assay can be used to detect DNA single strand breaks in individual cells. A modified version of the comet assay enables quantitation of DNA damage in individual cells by embedding lysed cells in agarose on slides. Cells are lysed by detergent and high salt allows DNA denaturation and unwinding. At high pH, DNA breaks will be revealed. Fragmented DNA molecules carry a negative charge and migrate towards the anode in an electric field. DNA is visualised by staining by ethidium bromide or propidium iodide. The size of DNA fragments and the number of breaks determine the migration and pattern of the comet seen. Some thoughts suggest DNA is not migrating as fragments but the broken ends of DNA are pulled out (Collins *et al.*, 1997). Tail DNA content or tail moment (product of tail length and tail DNA content) increases with damage which can be quantified by computer image analysis (e.g. Komet 3.0, Kinetic Image Analysis, Liverpool). Single stranded breaks can be distinguished from doubled stranded breaks by alkaline conditions for lysis and electrophoresis, since DNA duplex is denatured at alkaline pH (Olive *et al.*, 1990).

The comet assay is now suggested for use as a biomonitoring tool. For example, assessing occupational exposure to carcinogens and radiation, endogenous damage, disease states (Tice & Strauss, 1995; Collins *et al.*, 1997). It can also be used to study the *in vitro* and *in vivo* genotoxic or genoprotective

effect of certain agents (Anderson *et al.*, 1995; Collins *et al.*, 1997; Duthie *et al.*, 1997; Panayiotidis & Collins, 1997).

Summary And Questions To Be Addressed By Study

The human body is equipped with various antioxidants which oppose the effects of ROS and RNS. However, if deficient for any reason, for example, dietary deficiency, oxidative tissue damage may follow. On the other hand, if antioxidant defence can be improved, oxidative changes may be slowed or even prevented, and the risk of disease lowered. Many members of the *in vivo* antioxidant defence team are endogenous compounds. However, dietary input of antioxidants such as the vitamins C and E is important. Other possibly important dietary antioxidants include polyphenols which can be found in plant based foods, and in beverages such as tea and red wine. In addition, some kinds of Chinese herbal medicine and western therapeutic drugs are reported to have antioxidant activity. However, under certain conditions, antioxidants may have pro-oxidant activity, and the bioavailability of plant-based antioxidants is unclear. To be effective, antioxidants must be present in high enough concentration and reach the site required to oppose reactive species. Understanding the relative antioxidant properties and systemic effects on antioxidant status of dietary agents, such as tea, wine, and Chinese herbal medicines, has important implications with regard to developing cost-effective dietary approaches for lowering risk of diseases associated with oxidative stress.

The questions to be addressed by this study, therefore, were:

1. Is there any antioxidant activity in certain medicinal agents such as oral hypoglycaemic and lipid lowering drugs?
2. What is the antioxidant power in certain foods and beverage such as vegetables, fruits and teas, which are rich in antioxidant vitamins or flavonoids?
3. Can this antioxidant power in foods or beverages be absorbed and enter the systemic circulation?
4. Is there any protective effect of these individual antioxidants or dietary agents on DNA in human cells?
5. Is there any evidence that antioxidants can exert pro-oxidant, damaging effects?

CHAPTER 3 ANTIOXIDANT ACTIVITIES OF SELECTED DRUGS: A PILOT STUDY

Introduction

Hypercholesterolaemia is an established risk factor for development of atherosclerosis, however atherosclerosis and premature coronary heart disease can develop in the absence of increased plasma cholesterol. This may be owing to oxidation of low density lipoprotein (OxLDL). Formation of OxLDL may be a major factor for accelerated atherogenesis (Steinberg *et al.*, 1989; Benzie, 1996a). Diabetes mellitus is also associated with depleted antioxidant defence status and increased oxidative stress (Maxwell & Lip, 1997; Strain & Benzie, 1998a). Duration and status of metabolic control of diabetes are related to oxidative stress (Singh *et al.*, 1997). Diabetes mellitus is associated with dyslipidaemia and an increased risk of coronary heart disease, which is an important cause of death in NIDDM (Laakso *et al.*, 1993). Some drugs have been reported to have antioxidant potential (Benzie & Tomlinson, 1998). If confirmed, these drugs may offer additional therapeutic benefit in diseases associated with increased antioxidant stress.

Aims

The aim of this pilot study was to perform initial screening of antioxidant power of two groups of drugs, oral hypoglycaemic and lipid lowering agents, in order to explore possible additional therapeutic effects on oxidant / antioxidant

balance. This was achieved by measuring and comparing the *in vitro* antioxidant power of the drug extracts.

Materials And Methods

Seven oral hypoglycaemic drugs and ten lipid lowering drugs (Tables 3.1 & 3.2) were extracted into distilled water, 0.05 M HCl (Merck), 0.05 M NaOH (Riedel-de Haen) and 100% ethanol (Riedel-de Haen) separately. Drugs tested were provided by Professor Brian Tomlinson (Chinese University of Hong Kong, Hong Kong). Each tablet or the contents of each capsule of each drug, as provided, was ground into fine powder before the addition of 5 or 10 ml of solvent. This thoroughly mixed mixture was then transferred from the mortar into a clean glass container which was capped and then placed on the roller mixer for 10 minutes mixing at room temperature. Supernatant was obtained by centrifugation at 3700 rpm for 15 min. The antioxidant power of the extracts was determined by the Ferric Reducing /Antioxidant Power Assay (FRAP), using a 10 min reaction time. The FRAP assay was performed as previously described (Benzie & Strain, 1996b; Benzie & Tomlinson, 1998; reagent preparation and Cobas Fara programme, see Appendices 1 and 2) using a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd., Basel, Switzerland). In the FRAP assay, antioxidants in the sample reduce Fe^{3+} /tripyridyltriazine complex, which is present in excess to the blue coloured product. The increase of absorbance at 593 nm can then be measured and the change of absorbance is proportional to the total ferric reducing/antioxidants power. In the test procedure, 10 μl of sample was added to 300 μl FRAP reagent and the reaction was carried out at 37°C. The 10 min absorbance change was determined and compared to that of the 1000 $\mu\text{mol/l}$ FeSO_4 standard solution. The

FRAP assay has a limit of detection of $<2 \mu\text{mol/l}$ reducing / antioxidant power and precision is excellent; within- and between- run CVs are $<0.5\%$ and 1.0% respectively at between 500 and $2000 \mu\text{mol/l}$ antioxidant/reducing power, $n>8$ in each case. For those drugs with significant measured antioxidant activity, the extracts were tested again with FeCl_3 -free FRAP reagent to exclude any artefact in results owing to free Fe^{2+} in the extract.

Results are presented in terms of μmol FRAP per tablet or capsule, in a typical daily dosage of each preparation tested, and as $\mu\text{mol}/\mu\text{mol}$ active pharmaceutical ingredient. It should be noted however, that resource limitations meant that actual drug concentrations of the specific active ingredients, i.e. the individual drug, was not measured. Where results are expressed as μmol FRAP per tablet, capsule or daily dose, results are likely to be a combination of the drug of interest plus components in the packing material. Where results are expressed as μmol FRAP/ μmol or mg drug, this has used, for the purposes of this pilot study, an assumption of drug concentration based on stated amount of active ingredient in the tablet/capsule and the volume of solvent used in extraction.

Calculation of FRAP value:

$$\frac{10 \text{ min absorbance change of test sample}}{10 \text{ min absorbance change of standard}} \times 1000 \mu\text{mol/l} *$$

*(FRAP equivalence value of $1000 \mu\text{mol/l}$ Fe^{2+} std)

Results

Gliclazide was the only hypoglycaemic drug tested which showed significant activity in the FRAP assay. A linear concentration-response

relationship seen for both the NaOH and ethanolic extracts of gliclazide up to about 500 mg/l (Figure 3.1). Since endogenous Fe^{2+} in the extract, which is not relevant to the total antioxidant activity of the drug may also react and give apparently high FRAP value, FeCl_3 -free FRAP reagent was used to exclude any artefact from Fe^{2+} . The contribution of Fe^{2+} to the FRAP value of extracts of gliclazide was found to be less than 0.5%. Other hypoglycaemic drugs tested had negligible antioxidant activity (less than 10 μmol per tablet or capsule) (Table 3.1). The reaction of gliclazide was quite slow and did not reach end point within 12 minutes (Figure 3.2). The stoichiometric factor of gliclazide in NaOH extract was 3 (mean=2.89, SD=0.34, n=5) and in ethanolic extract was 2 (mean=2.15, SD=0.05, n=6) (Tables 3.3 & 3.4).

The lipid lowering drugs simvastatin and probucol showed high activities in the FRAP assay (Table 3.2). Other tested lipid lowering agents showed negligible antioxidant power (Table 3.2). Simvastatin showed linear concentration-response relationship up to 1000 mg/l (Figure 3.3). The reaction of simvastatin was rapid (Figure 3.4). About 9 % of the FRAP value in simvastatin was due to Fe^{2+} in the extract. The stoichiometric factor of simvastatin in water extract was 1 (mean=1.11, SD=0.04, n=5) (Table 3.5). Probucol had a linear concentration-response relationship up to about 1250 mg/l in ethanol (Figure 3.5). The reaction of probucol was slow and did not reach end point within 12 min. About 17 % of the FRAP value of probucol extract was due to the presence of Fe^{2+} . The stoichiometric factor of the ethanolic probucol extract was less than 1 (mean=0.48, SD=0.18, n=5) (Table 3.6).

Discussion

Gliclazide has been reported to possess antioxidant activity, specifically by scavenging $O_2^{\cdot-}$, $\cdot OH$ and NO^{\cdot} (Noda *et al.*, 1997). Treating diabetic patients with gliclazide reportedly can lower plasma lipid peroxides, an indicator of oxidative stress (Desfaits *et al.*, 1998). In this current study, alkaline and ethanolic extracts of gliclazide tablet were found to possess high antioxidant power. Gliclazide is reportedly not soluble in water, but to be slightly soluble in ethanol (British Pharmacopoeia Commission, 1998). The antioxidant power measured in this pilot study, therefore, is likely to be a combined contribution of packing material and the active ingredient (gliclazide). Although lactose and glucose which are the main packing material are not reactive in FRAP assay, some of the drugs are known to contain antioxidant as preservative such as butylated hydroxyanisole can react. Drugs may become hydroxylated *in vivo*, and this can lead to increased antioxidant power because of the additional hydroxyl group. This may be the reason why the NaOH extract of gliclazide had higher antioxidant power than the ethanolic extract. A typical daily dosage of gliclazide is two 80 mg tablets which is equivalent to about 1450 μmol FRAP (Table 3.7). In comparison, this is the same amount of antioxidant power in 128 mg ascorbic acid.

Simvastatin is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor and is reportedly insoluble in water and is soluble in ethanol (RxList, 1997). A lower antioxidant power was seen in the ethanolic extract of simvastatin than in water and acid extracts. This indicates that the antioxidant power of water and acid extracts may be derived from the packing material. While that of the ethanol extract may be related to the activity of the drug itself.

Simvastatin has been reported to lower the plasma ubiquinone level (Watts *et al.*, 1993). Ubiquinone, present in LDL as an antioxidant and shares the same *in vivo* synthetic pathway as cholesterol. Therefore inhibition of cholesterol synthesis by statins, such as simvastatin, may also inhibit ubiquinone production and hence lower resistance of LDL to oxidation. However, no adverse effect of simvastatin on ubiquinone was found in a later study (Laaksonen *et al.*, 1996). The possibility of compensation by the antioxidant property of simvastatin in LDL is worth considering.

Although the antioxidant power of simvastatin may be low, the antioxidant power of the packing materials should not be ignored. The usual dosage of simvastatin is two 10 mg tablets daily which is equivalent to about 52 μmol FRAP, or 4.5 mg pure vitamin C (Table 3.7).

Probucol is a lipid lowering agent which can decrease serum LDL and has known antioxidant properties (Scott, 1997). Increased activity of antioxidant enzymes and increased resistance to LDL oxidation after treatment with probucol have been reported (Kaul *et al.*, 1995; Mantha *et al.*, 1996; McDowell *et al.*, 1995). Besides the LDL lowering action of probucol, it has been reported to reverse the antioxidant and functional deficit in diabetic-induced myocardial changes, for example restoration of SOD, GSH-Px activities and blood pressure (Kaul *et al.*, 1996). The stoichiometric factor of probucol was less than one in this current study. This is likely to be due to the slow reaction rate in the FRAP assay and/or the incomplete extraction of drug in the solvent. An estimated 890 μmol FRAP is ingested with a daily dose of probucol of four 250 mg tablets. This is equivalent to

the antioxidant power of 78 mg ascorbic acid (Table 3.7). It should be noted, however, that owing to its significant HDL-lowering effects, probucol has been withdrawn from the US market.

Owing to the low solubility of simvastatin in water, the antioxidant power measured in the water extract was probably derived from the packing material. It was found that antioxidant butylated hydroxyanisole was added into the tableting material of simvastatin, presumably as a preservative (RxList, 1997). This may be true for other drug preparations also, although no information has been found with regard to this from the information made available by the manufacturers. Nevertheless, any antioxidants added to these drugs are ingested along with the active pharmaceutical ingredient. Although the antioxidant power of the tablet may not be due to the active ingredient itself, therefore, additional beneficial effect could nevertheless be derived from both antioxidant preservative and active ingredient. It may be worth considering the antioxidant power of the tablet/capsule when choosing one from a list with similar primary pharmaceutical effect.

The nutrient reference range or recommended daily allowance (RDA) of vitamin C is 40 or 60 mg respectively. Daily ingestion antioxidant power from the RDA of vitamin C is 450-680 μmol FRAP, while that from the RDA of vitamin E (10 mg), is equivalent to about 48 μmol FRAP. It should be noted that comparison of drugs and vitamins has been made in terms of antioxidant power only, and does not imply a suggestion of the replacement of vitamins or their function with the drugs tested. Vitamins C and E have specific functions but they are likely to be mediated largely through their antioxidant properties (Bode, 1997; Benzie, 1999).

The antioxidant power of gliclazide, simvastatin and probucol tablets obtained from the typical daily dosage is not low when compared to the RDA of vitamins C and E. Furthermore drugs may be target specific. For example, probucol is taken up by the adipose tissue (Reynolds, 1993), so that the antioxidant effect may be exerted on a specific organ, tissue or structure to give local area protection. Some of the drugs, e.g. carvedilol, become active after they are metabolised by the body, and the metabolites of the drug may possess antioxidant effect (Benzie, personal communication). Some people are on several drugs for many years. The possible cumulative and additive effects should be taken into account. Study of the *in vivo* antioxidant power effect during treatment would be useful to further investigate a possible beneficial role of medicinal agents with antioxidant properties.

In summary, this pilot study showed that gliclazide, simvastatin and probucol possess significant level of antioxidant power in the preparations provided. Owing to the limitation of resources, the actual drug concentration in the extracts could not be measured, and the total antioxidant power in extracts of each tablet or capsule of each drug was studied. Further study on the purified drugs and metabolites of drugs is essential for the possible pleiotropic benefits in term of antioxidant effect to be established.

Data and information presented in this chapter have been published in:

Antioxidant potential of lipid lowering drugs *in vitro*. *Journal of the Hong Kong College of Cardiology* (1998), 6 (1), 51. (with IFF Benzie, B Tomlinson)

Table 3.1 Antioxidant power ($\mu\text{mol FRAP}$) in hypoglycaemic drugs in different solvent extractions. Each result represents the mean of triplicate measurement of each extraction.

Hypoglycaemic Agent	Mol. Mass	$\mu\text{mol FRAP per tablet/capsule}$			
		Water	HCl	NaOH	Ethanol
Metformin HCl (Brand A), 500 mg	165.6	*	*	*	*
Metformin HCl (Brand B), 500 mg	165.6	*	*	*	*
Chlopropamide, 250mg	276.7	*	*	*	*
Gliclazide, 80 mg	323.4	*	*	740	543
Glipizide, 5 mg	445.5	*	*	*	*
Tolbutamide, 500 mg	270.4	*	*	*	*
Glibenclamide, 5 mg	494	*	*	*	*

* = less than 10

Table 3.2 Antioxidant power ($\mu\text{mol FRAP}$) in lipid lowering drugs in different solvent extractions. Each result represents the mean of triplicate measurement of each extraction.

Lipid Lowering Agent	Mol. Mass	$\mu\text{mol FRAP per tablet/capsule}$			
		Water	HCl	NaOH	Ethanol
Fluvastatin sodium, 40 mg	433.5	*	*	*	*
Simvastatin, 10 mg	418.6	23	26	*	14
Pravastatin sodium, 10 mg	424.5	*	*	*	*
Lovastatin, 20 mg	404.5	*	*	*	*
Acipimox, 250 mg	154.1	*	*	*	*
Bezafibrate, 400 mg	361.8	*	*	I	*
Gemfibrozil, 900 mg	250.3	*	*	I	I
Fenofibrate, 300 mg	260.8	*	*	*	I
Probucol, 250 mg	519.6	*	*	*	223
Atorvastatin calcium, 10 mg	1209.4	*	*	*	*

* = less than 10

I = interference, precipitate formed during reaction

Table 3.3 FRAP values of gliclazide in different concentrations (NaOH extraction). Each result represents the mean of triplicate measurement of a single extraction.

Gliclazide Conc. ($\mu\text{mol/l}$)	FRAP ($\mu\text{mol/l}$), Mean (SD)	$\mu\text{mol FRAP}/\mu\text{mol}$ Gliclazide
123.7	403 (11)	3.26
247.4	769 (22)	3.11
494.8	1481 (49)	2.99
1098.4	2913 (71)	2.65
1648.2	4025 (80)	2.44

Table 3.4 FRAP values of gliclazide in different concentrations (ethanol extraction). Each result represents the mean of triplicate measurement of a single extraction.

Gliclazide Conc. ($\mu\text{mol/l}$)	FRAP ($\mu\text{mol/l}$), mean (SD)	$\mu\text{mol FRAP}/\mu\text{mol}$ Gliclazide
31.0	66 (2)	2.13
61.9	131 (4)	2.12
123.7	258 (5)	2.09
247.4	535 (10)	2.16
618.5	1362 (37)	2.20
1237.0	2716 (43)	2.20

Table 3.5 FRAP values of simvastatin in different concentrations (water extraction). Each result represents the mean of triplicate measurement of a single extraction.

Simvastatin Conc. ($\mu\text{mol/l}$)	FRAP ($\mu\text{mol/l}$), Mean (SD)	$\mu\text{mol FRAP}/\mu\text{mol}$ Simvastatin
478	555 (24)	1.04
956	1014 (13)	0.95
1434	1597 (18)	1.00
1912	2118 (31)	1.00
2390	2613 (16)	0.98

Table 3.6 FRAP values of probucol in different concentrations (ethanol extraction). Each result represents the mean of triplicate measurement of a single extraction.

Probucol Conc. ($\mu\text{mol/l}$)	FRAP ($\mu\text{mol/l}$), Mean (SD)	$\mu\text{mol FRAP}/\mu\text{mol}$ Probucol
150	42 (7)	0.28
301	102 (22)	0.34
601	267 (53)	0.44
1203	764 (107)	0.64
2406	1675 (151)	0.70

Table 3.7 Antioxidant power ($\mu\text{mol FRAP}$) in hypoglycaemic and lipid lowering agents in typical daily dose.

Hypoglycaemic / Lipid Lowering Agents	Typical daily dose in μmol FRAP	$\mu\text{mol FRAP per } \mu\text{mol active ingredient}$			
		Water	HCl	NaOH	Ethanol
Gliclazide	1450	-	-	3.0	2.1
Simvastatin	52	1.0	1.0	-	0.5
Probucol	890	-	-	-	0.5

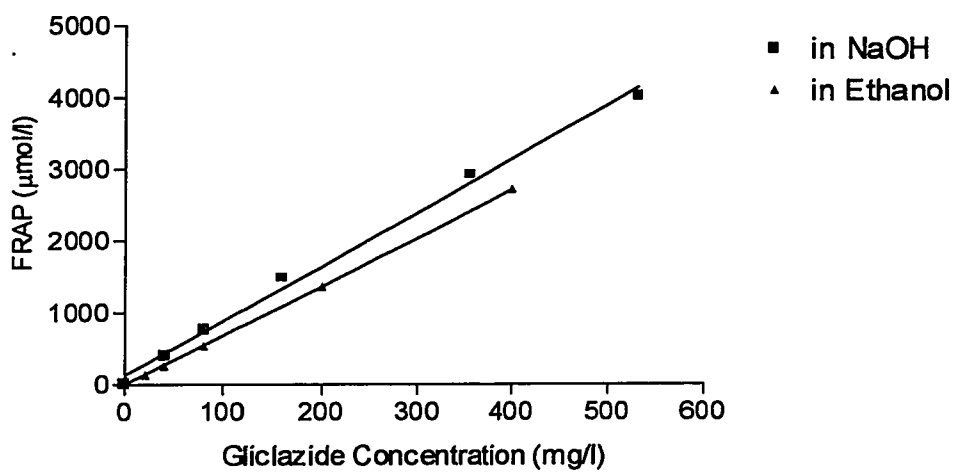


Figure 3.1 Concentration-response line of gliclazide in 0.05 M NaOH and 100% ethanol (n=3, 1 SD error bars). Showing a linear dose-response up to a (nominal) gliclazide concentration of 500 mg/l.

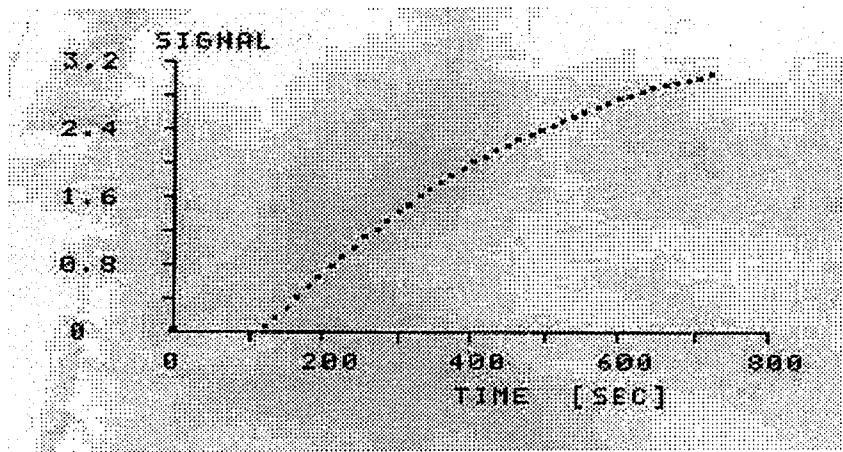


Figure 3.2 Reaction kinetics of gliclazide in NaOH. Result showed that the reaction was fairly slow.

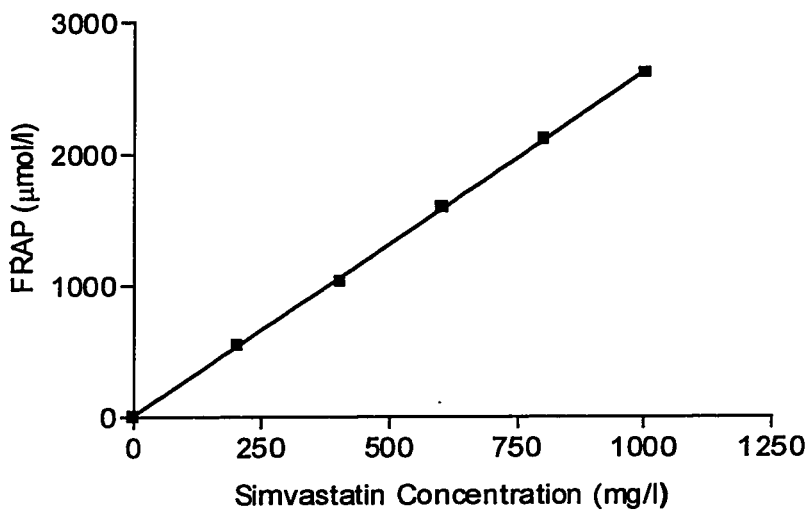


Figure 3.3 Concentration-response line of simvastatin in water (n=3, 1 SD error bars). Showing a linear dose-response up to a (nominal) simvastatin concentration of 1000 mg/l.

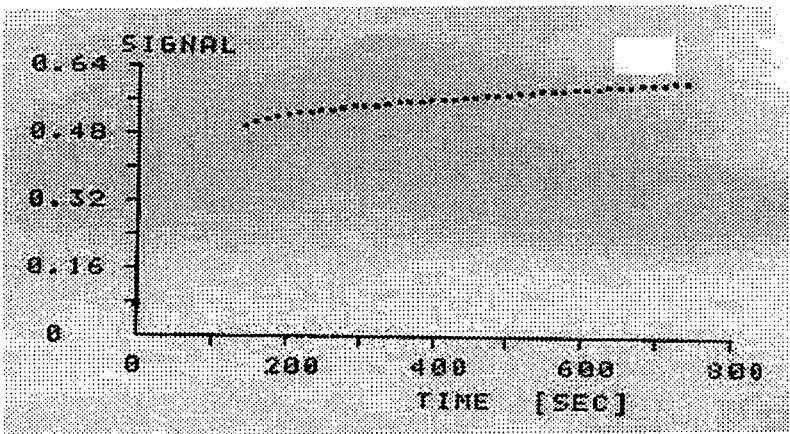


Figure 3.4 Reaction kinetics of simvastatin in water. Result showed that the reaction was rapid.

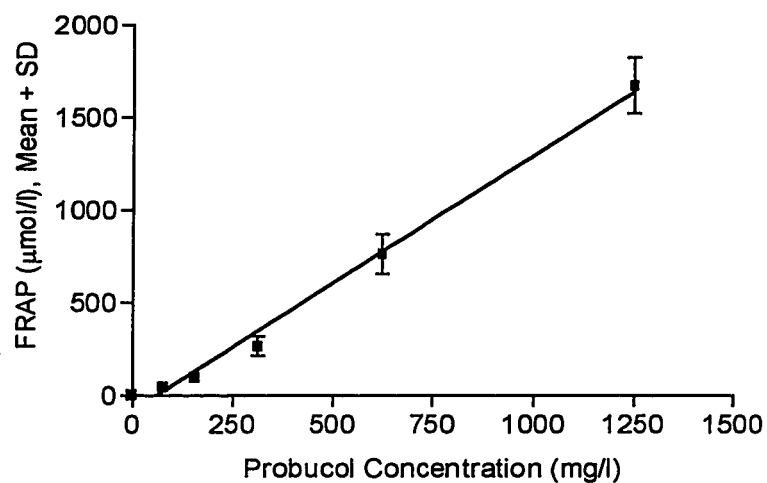


Figure 3.5 Concentration-response line of probucol in ethanol (n=3, 1 SD error bars). Showing a linear dose-response up to a (nominal) probucol concentration of 1250 mg/l.

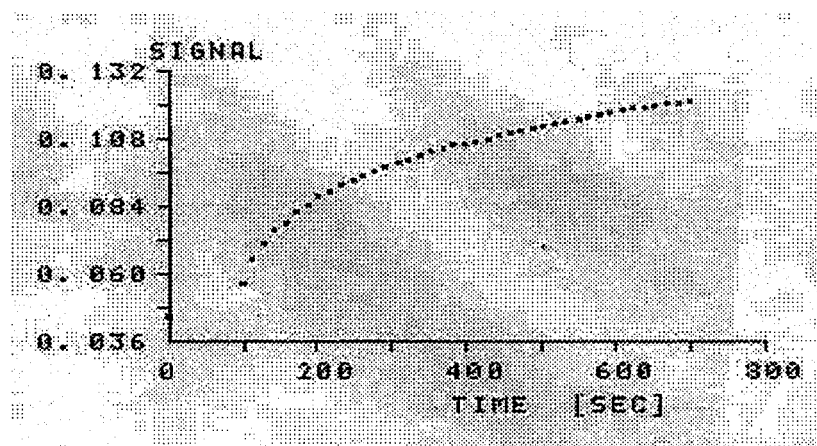


Figure 3.6 Reaction kinetics of probucol in ethanol. Result showed that the reaction was slow.

CHAPTER 4 *IN VITRO* ANTIOXIDANT ACTIVITIES AND ASCORBIC ACID CONTENT OF FRUITS AND VEGETABLES BY THE FRASC ASSAY

Introduction

Diets rich in fresh fruits and vegetables are protective against chronic, degenerative disease, such as cancer and coronary heart disease (CHD) (Hansson *et al.*, 1993; Kushi *et al.*, 1996; Steinmetz, 1996; Gey, 1998). Although it is unlikely that reactive oxygen species (ROS) are the sole cause of cancer, observations imply that ROS promote carcinogenesis and increase the risk of several types of cancer (Machlin, 1995). Epidemiological findings show that high consumption of antioxidant-rich foods is associated with a reduction in risk of all cancers (Gey, 1993; Benzie, 1998). High intake of carotene-rich fruits and vegetables is also associated with lower cancer risk, although the benefits and role of β -carotene are unclear (Weisburger, 1991; Hennekens *et al.*, 1996; Omenn *et al.*, 1996). A large cross-cultural epidemiological study by Gey and co-workers demonstrated a strong inverse relationship between heart disease mortality and plasma vitamin E levels (Gey *et al.*, 1991), and dietary supplementation with the antioxidant vitamin C and E reportedly lowers the risk of cataract (Muggli, 1993).

Beneficial effects of diets rich in fruits and vegetables are thought to be owing, at least in part, to plant-based antioxidant compounds, such as ascorbic acid, carotenoids and flavonoids (Strain & Benzie, 1998b). The effects on health may be due to the inhibition of nitrosoamine formation and to improved systemic

antioxidant status (Borenstein, 1987). To minimize risk of chronic degenerative disease, five or more servings fruits and vegetables daily are recommended (World Cancer Research Fund, 1997). Most individuals do not meet this dietary target, however. Also, the development of genetically modified fruits and vegetables may result in a different antioxidant profile or content. In helping to design diets which might permit enhanced antioxidant status at more realistic quantities of intake, and to assess the effect of genetic modification, it would be useful to know the 'antioxidant power' and individual antioxidant content of various plant-based foods. Food tables also require updating with regard to previously unstudied varieties of fruits and vegetables.

Aims

The aim of this part of the study was to measure the 'total antioxidant power', and the ascorbic acid content of a range of fresh, uncooked fruits and vegetables using a newly developed, automated technique (Benzie & Strain, 1997b; Benzie & Strain, 1999), in order to assess their potential for improving *in vivo* antioxidant status. In addition, variability in antioxidant power and ascorbic acid content within the same type of fruit or vegetable purchased on different occasions was investigated.

Materials And Methods

Sample preparation:

Extracts of washed, uncooked, fresh fruits and vegetables were prepared by homogenizing 5 g fresh weight (with or without skin and seeds as would

normally be eaten) in 100 ml distilled water for 30 seconds. Homogenates were filtered, and the ferric reducing/antioxidant power (FRAP) value (US patent pending) (Benzie & Strain, 1996b) and ascorbic acid concentrations were measured in triplicate immediately afterwards. FRAP and ascorbic acid measurements were combined in one test, referred to as FRASC (Benzie & Strain, 1997b; Benzie & Strain, 1999). Water extracts of vegetables showed a near neutral pH, and as ascorbic acid is more stable in acidic medium, acetate buffer (75 mmol/l, pH 3.6) was also used for extraction of vegetables. Results on water and buffer extracts were compared. Seventeen varieties of fruits and 17 different types of vegetables were tested; for each fruit and vegetable at least 3 samples, purchased from local shops on different days, were tested. In all cases measurements were performed within two days of purchase, with fruits and vegetables stored intact at 4°C and in the dark until extracted and assayed.

The FRASC assay

The FRASC assay was performed as previously described (Benzie & Strain, 1997) using a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd., Basel, Switzerland) (for details and Cobas Fara test programme, see Appendices 1 & 2). In the FRASC assay, reductants ('antioxidants') in the sample reduce a ferric / tripyridyltriazine complex, present in stoichiometric excess, to the blue coloured ferrous form. The timed change of absorbance at 593 nm is proportional to the combined (total) ferric reducing / antioxidant power (FRAP value) of the antioxidants in the sample. In the FRASC assay, ascorbic acid is selectively destroyed by the addition of ascorbate oxidase (Sigma, USA) solution to one of a pair of sample aliquots (40 µl of 4 IU/ml solution added to 100 µl sample). In this

case the 60 seconds absorbance change in the FRAP assay of a sample to which ascorbate oxidase was added is subtracted from the absorbance of a matching aliquot to which water, rather than ascorbate oxidase was added; the difference is due to ascorbic acid. The 4 min absorbance change of the aliquot diluted in water is owing to the 'total antioxidant power' in sample.

Calculation:

For FRAP value:

$$\frac{\text{4 min absorbance change of test sample}}{\text{4 min absorbance change of standard}} \times 1000 \mu\text{mol/l} *$$

*(FRAP equivalence value of 1000 $\mu\text{mol/l Fe}^{2+}$ std)

For ascorbic acid:

$$\begin{aligned} & \text{1 min absorbance change of test sample with water only} - \\ & \text{1 min absorbance change of test sample with ascorbate oxidase} \\ & = \text{net absorbance change due to ascorbic acid} \end{aligned}$$

$$\frac{\text{net absorbance change due to ascorbic acid}}{\text{1 min absorbance change of standard}} \times 500 \mu\text{mol/l} **$$

**FRAP equivalence value of 1000 $\mu\text{mol/l Fe}^{2+}$ std. 500 μM of ascorbic acid gives 1000 $\mu\text{mol/l}$ FRAP value when Fe^{2+} is used as standard

The precision of ascorbic acid measurement is good, within- and between run CVs are <5% at 25, 50, 100, and 440 $\mu\text{mol/l}$. Precision of FRAP is excellent; within- and between- run CVs are <0.5% and 1.0% respectively at between 500 and 2000 $\mu\text{mol/l}$ antioxidant/reducing power, $n > 8$ in each case.

In vitro antioxidant power results are expressed as μmol ferric reducing / antioxidant power (the FRAP value) per 100 g of fresh wet weight. Ascorbic acid results are expressed as mg per 100 g of fresh wet weight. Data are presented as

the mean (SD) of triplicate measurements on each of 3 separate samples of each fruit or vegetable.

Results

Results for both parameters of interest covered a wide range (Tables 4.1 and 4.2). Of the fruits tested, the highest antioxidant power was in strawberries (1594 $\mu\text{mol}/100\text{ g}$). The lowest value was in Chinese pears (146 $\mu\text{mol}/100\text{ g}$). Ascorbic acid content was also highest in strawberries (54 mg/100 g). Apples, pears and bananas were low in ascorbic acid. Of the vegetables tested, choy sum (a Chinese green leafy vegetable) contained the highest antioxidant power (1042 $\mu\text{mol}/100\text{ g}$); lowest was iceberg lettuce (88 $\mu\text{mol}/100\text{ g}$). Ascorbic acid content of vegetables was high in cauliflower (30 mg/100 g) and in the Chinese vegetables choy sum, pak choy, wombok and spring onion. Different samples of fruits and vegetables, particularly persimmon, grape, wombok, green turnip, carrot and cauliflower, which were purchased on different days of the same season, showed fairly wide variation in antioxidant power. This was probably caused by different age and conditions of storage. When results obtained on the near neutral pH water extracts and acidic acetate buffer extracts of vegetables were compared, the ascorbic acid and antioxidant content of most vegetables was found to be higher in the acidic extracts ($p < 0.05$, paired t test)(Table 4.2).

Discussion

The higher ascorbic acid content of the acidic vegetable extracts is likely to be owing to improved stability of ascorbic acid at acidic pH. The higher antioxidant power of the acidic vegetable extracts is owing, at least in part, to their

higher ascorbic acid content. Results obtained in this study were generally lower than that reported in published food tables of McCane & Widdowson's (Holland *et al.*, 1991). This was likely to be due to the extraction procedure of ascorbic acid in metaphosphoric acid, which is a very strong acid (Helrich, 1995). The preservation of ascorbic acid by metaphosphoric acid is unrealistic in daily food processing and may have include dehydroascorbic acid, however that is the reason water and a less acidic buffer were used for extraction in this experiment. It is also possible that enzymes such as polyphenol oxidase are less active at acidic pH. Polyphenol oxidase is found in many plants (Martinez & Whitaker, 1995; Das *et al.*, 1997), and is responsible for the browning seen in bruised, fragmented or chopped fruit, and for the change, from green to black, which occurs in crushed or chopped tea leaves. The antioxidant power of green tea is much higher than that of black tea because of enzymatic oxidation of polyphenolic tea antioxidants (Weisburger, 1996; Benzie & Szeto, 1999, for details, see Chapter 5). It is possible that a similar loss of phenolic-related antioxidant power in vegetables may occur with chopping or pureeing during food processing. It is worth noting that this current study used freshly prepared extracts. The loss of ascorbic acid, and possibly other antioxidants, is clearly rapid in chopped vegetables. This has implications with regard to the dietary intake of antioxidants in processed vegetables, as the content of the food as eaten may be considerably less than that of the fresh whole food.

There have been few published reports on the antioxidant potential of fruits and vegetables to date, although published tables containing data on vitamin C content of foods are available. Food tables require constant updating, however.

Tropical and oriental varieties of fruits and vegetables, previously rarely found in western diets, are becoming available throughout the world; previously seasonal fruits and vegetables are now available year-round; genetically modified fruits and vegetables are being developed on a regular basis. In addition, it is not always clear whether published data relate to the amount of the reduced form (ascorbic acid) only, or to the total amount of reduced plus oxidised (dehydroascorbic acid) vitamin C. Ascorbic acid, the reduced form, was measured in this study, as it is likely that this is the more physiologically relevant form. As can be seen from Tables 4.1 and 4.2, a single portion of fresh fruit or uncooked vegetables can provide considerably more than the current recommended daily intake 40 mg ascorbic acid (Department of Health, 1991). Some fresh fruits and vegetables, however, have negligible ascorbic acid content. Furthermore, results of this study indicate that the ascorbic content of fruits and vegetables may decrease rapidly and significantly after chopping, shredding or pureeing. These results have implications for dietary planning and food preparation if ascorbic acid intake is to be optimised (Benzie, 1999).

With reference to the antioxidant power of fruits and vegetables, Figures 4.1 to 4.4 present a comparison between the results of this current study and those retrieved from the only (to the author's knowledge) previously published reports (Cao *et al.*, 1995; Wang *et al.*, 1996). These two studies used a different method of assessing total antioxidant power, and sample preparation was also different. Results, therefore, are compared in terms of rank order, as well as absolute antioxidant values. Good agreement was seen for most varieties of fruits tested by both methods, but not for vegetables, most likely owing to differences in sample

processing. Water and acetone were used for extraction, and pH 7.0 phosphate buffer was used for making dilutions in the ORAC assay. The method (FRASC) used in this current study, however, has the advantage of being able to measure ascorbic acid content at the same time as antioxidant power, and is simple and rapid. A short assay time and a simple procedure enable a more 'real time' picture of antioxidant power and ascorbic acid content of fruits and vegetables to be obtained.

In conclusion, this part of the study presents additional data on the antioxidant power and ascorbic acid content of a range of fruits and vegetables, including some Chinese varieties not previously studied. The method used enables rapid analysis of freshly prepared food extracts. Results indicate that different types of fruits and vegetables have widely different levels of antioxidant content, and that storage and processing effects can lead to significant decreases in antioxidant power and ascorbic acid content. While diets rich in fruits and vegetables are beneficial to health, the diet of most individuals does not meet, or even approach, the target level of five portions of fruits and vegetables per day. If it is confirmed that the health benefits of fruits and vegetables are mediated through their antioxidant content, optimising antioxidant intake would be a primary aim in preventive medicine. Optimising intake, however, requires data on the antioxidant content of fruits and vegetables as they are eaten. Existing food tables require updating, therefore, to include the antioxidant content and profile of fresh, stored, processed and cooked fruits and vegetables, as well as data on tropical, oriental, and genetically modified varieties not previously studied. Diets incorporating fruits and vegetables with the highest antioxidant content could then

be planned, and methods of food preparation and storage which minimise antioxidant loss could be used. The end result could be a more realistic and achievable dietary target of daily servings of fruits and vegetables, with the realisation of potential health benefits.

Data and information presented in this chapter have been submitted for publication in:

Total Antioxidant power and ascorbic acid content of fresh fruits and vegetables: implications for dietary recommendations. *Journal of Food Science* (with IFF Benzie)

Table 4.1 Total antioxidant power as FRAP and ascorbic acid (AA) content of various kinds of fruits in aqueous extraction (per 100 g fresh weight)

Fruit	μmol FRAP, Mean (SD), n≥3	mg AA , Mean (SD), n≥3	Proportion of antioxidant power contributed by AA (%)
Strawberry	1594 (278)	54 (6)	39
Lemon	1040 (148)	42 (7)	46
Plum	928 (224)	1 (1)	<1
Orange	942 (274)	33 (4)	40
Kiwi Fruit	820 (22)	52 (10)	73
Grapefruit (pink)	808 (30)	39 (1)	54
Persimmon	774 (502)	8 (6)	11
Apple (green)	630 (16)	ND	<1
Mandarine	540 (76)	24 (2)	50
Mango	506 (14)	21 (4)	48
Grape (green)	478 (230)	2 (1)	6
Apple (red)	420 (42)	ND	<1
Banana	420 (32)	ND	<1
Grape (red)	416 (98)	ND	<1
Pear	408 (108)	ND	<1
Pineapple	348 (142)	10 (2)	31
Chinese Pear	146 (44)	ND	<1

ND = not detected

Table 4.2 Total antioxidant power as FRAP and ascorbic acid (AA) content of various kinds of uncooked vegetables in aqueous and acetate buffer extractions (per 100 g fresh weight)

Vegetable	$\mu\text{mol FRAP}$, Mean (SD), $n \geq 3$		mg AA, Mean (SD), $n \geq 3$		Proportion of antioxidant power contributed by AA (%)	
	In water	In acetate buffer	In water	In acetate buffer	In water	In acetate buffer
Choy Sum	1042 (282)	1044 (204)	13 (6)	29 (14)	14	31
Pak Choy	620 (90)	844 (48)	9 (2)	28 (11)	17	38
Spring onion	590 (54)	804 (144)	15 (2)	28 (5)	30	39
Wombok	562 (276)	680 (420)	20 (8)	28 (17)	40	46
Peas (green)	490 (96)	446 (124)	ND	11 (1)	<1	29
Onion	432 (126)	288 (130)	8 (6)	5 (5)	22	19
Turnip (green)	358 (216)	372 (250)	14 (11)	15 (10)	44	45
Cabbage (long)	350 (72)	500 (112)	11 (4)	15 (6)	34	34
Broccoli	294 (56)	748 (246)	1 (2)	10 (4)	4	14
Cauliflower	284 (64)	588 (242)	14 (8)	30 (23)	56	59
Garlic	268 (100)	240 (38)	6 (5)	5 (2)	27	22
Tomato	236 (14)	312 (58)	12 (6)	9 (4)	58	32
Chinese lettuce	230 (46)	228 (144)	ND	1 (1)	<1	3
Carrot	166 (80)	240 (150)	1 (1)	1 (1)	5	7
Celery	156 (6)	134 (44)	ND	3 (1)	<1	27
Potato	144 (32)	232 (96)	ND	5 (2)	<1	26
Lettuce (iceberg)	88 (26)	58 (16)	ND	ND	<1	<1

ND = not detected

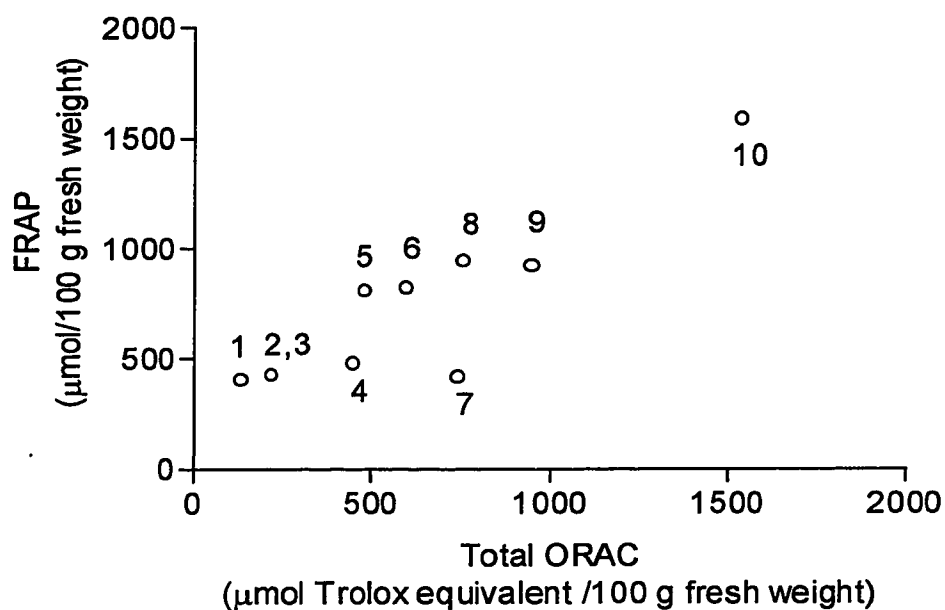


Figure 4.1 FRAP and ORAC values of 10 varieties of fruits. ORAC values were retrieved from previously published reports (Wang *et al.*, 1996). Results showed generally good agreement. Each point represents the mean of at least three samples in both FRAP & ORAC assays.

Legend

1	Pear
2	Apple
3	Banana
4	Grape, white
5	Grapefruit, pink
6	Kiwi Fruit
7	Grape, red
8	Orange
9	Plum
10	Strawberry

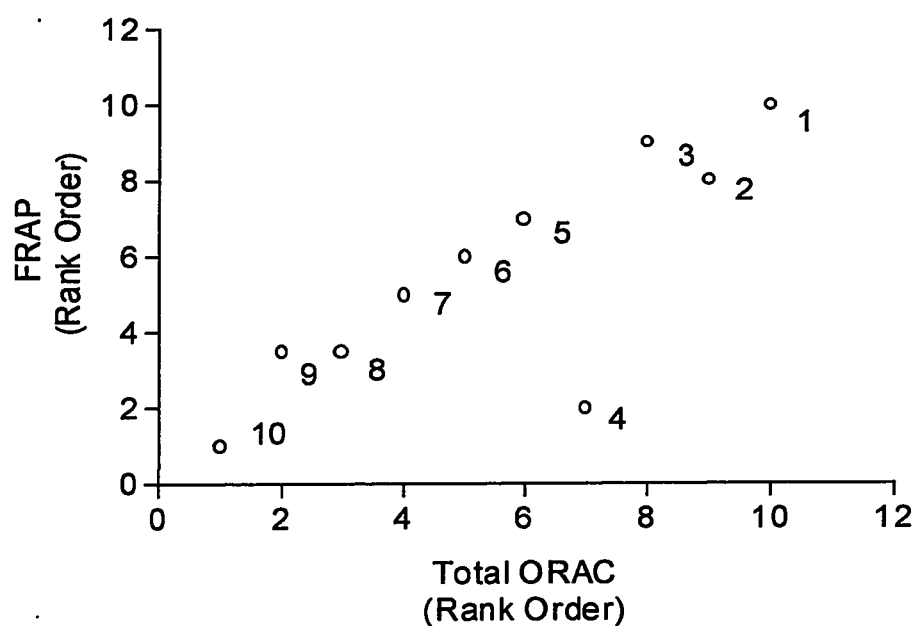


Figure 4.2 Comparison of FRAP and ORAC of 10 varieties of fruits in rank order. ORAC values were retrieved from previously published reports (Wang *et al.*, 1996). Results showed generally good agreement. Each point represents the mean of at least three samples in both FRAP & ORAC assays.

Legend

1	Pear
2	Apple
3	Banana
4	Grape, white
5	Grapefruit, pink
6	Kiwi Fruit
7	Grape, red
8	Orange
9	Plum
10	Strawberry

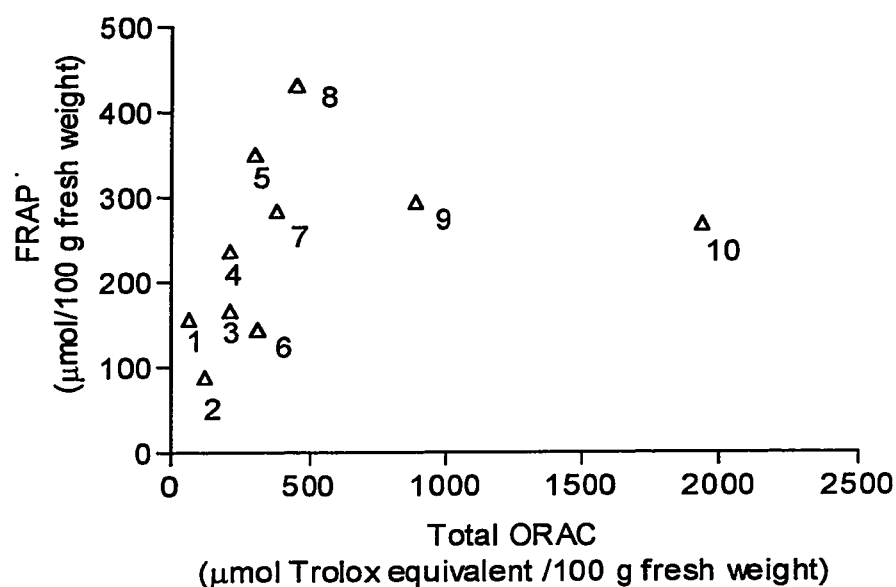


Figure 4.3 FRAP and ORAC values of 10 varieties of vegetables. ORAC values were retrieved from previously published report (Cao *et al.*, 1996). There was less agreement than with fruits, most likely owing to the different processing/extraction method used for vegetables for the ORAC assay. Each point represents the mean of at least three samples of both FRAC & ORAC assays.

Legend

1	Celery
2	Lettuce
3	Carrot
4	Tomato
5	Cabbage
6	Potato
7	Cauliflower
8	Onion
9	Broccoli
10	Garlic

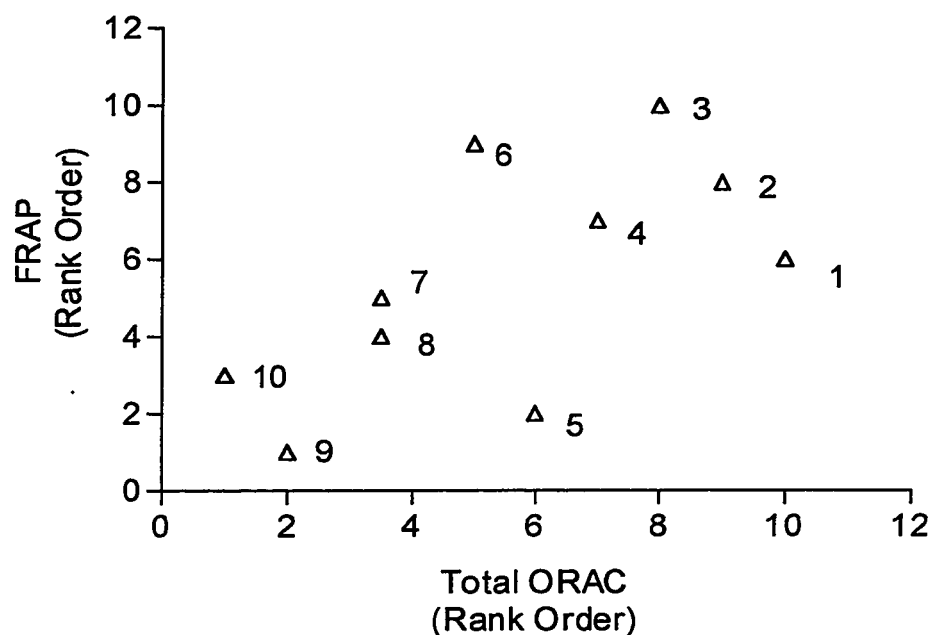


Figure 4.4 Comparison of FRAP and ORAC of 10 varieties of vegetables in rank order. ORAC values were retrieved from previously published report (Cao *et al.*, 1996). There was less agreement than with fruits, most likely owing to the different processing/extraction method used for vegetables for the ORAC assay. Each point represents the mean of at least three samples of both FRAC & ORAC assays.

Legend

1	Celery
2	Lettuce
3	Carrot
4	Tomato
5	Cabbage
6	Potato
7	Cauliflower
8	Onion
9	Broccoli
10	Garlic

CHAPTER 5 *IN VITRO* ANTIOXIDANT ACTIVITIES STUDY OF VARIOUS KINDS OF TEA BY THE FRAP ASSAY

Introduction

Tea (*Camellia sinensis*) is the most widely consumed beverage worldwide and has become an important agricultural product (Balentine, 1992). The type and quantity of tea taken varies in different countries and ethnic groups (Weisburger, 1996; Kohlmeier, 1997). Black ('fermented') tea is popular in the West; 'semi-fermented' Oolong-type tea is commonly drunk in Taiwan and parts of China; green ('non-fermented') tea is favoured in the rest of China, Northern Africa and Japan (Weisburger, 1996). Tea contains large amounts of polyphenolic compounds with antioxidant properties, and these may prevent oxidative damage of DNA (Zhao *et al.*, 1989; Scott *et al.*, 1993; Shiraki *et al.*, 1994; Cook & Samman, 1996; Rice-Evans *et al.*, 1996; Wiseman *et al.*, 1997; Zhang & Shen, 1997) and inhibit the peroxidation of low-density lipoprotein (Miura *et al.*, 1994; Ishikawa *et al.*, 1997; Luo *et al.*, 1997). Chemical changes such as oxidative damage and peroxidation are related to mutagenesis, increased risk of cancer (Emerit, 1994; Halliwell, 1996), and to atherogenesis and cardiovascular disease (Maxwell and Lip, 1997). Regular intake of tea therefore, may improve antioxidant status *in vivo* and thereby help lower risk of certain types of cancer and coronary heart disease (Stensvold *et al.*, 1992; Weisburger, 1996).

Tea antioxidants can protect against strong mutagens in animal models

(Yamane *et al.*, 1991; Hasegawa *et al.*, 1995; Leanderson *et al.*, 1997). Moreover, lower incidence of cancer in association with high consumption of tea has been reported in some epidemiological studies (La Vecchia *et al.*, 1992; Yang & Wang, 1993; Baron *et al.*, 1994; Gao *et al.*, 1994; Yu *et al.*, 1995). However, results are not consistent and a protective role for antioxidants in tea has not been clearly demonstrated (Goldbohm *et al.*, 1996; Kohlmeier *et al.*, 1997). This may be due to the form in which tea is taken (Kohlmeier, 1997). Different teas have different antioxidant compositions, and some tea antioxidants may be more active and / or more easily absorbed than others (Graham, 1992; Cook & Samman, 1996; Weisburger, 1996; Rice-Evans *et al.*, 1996; Van Acker *et al.*, 1996; Hollman, 1997; Kohlmeier, 1997).

Aims

The aim of this part of study was to measure and compare *in vitro* antioxidant activities of different types of tea to assess their relative potential for improving *in vivo* antioxidant status. The relationship of total phenolics content to antioxidant capacity in tea infusions was also studied and the stability of antioxidant potential in infusions of tea and the effect of dilution and effect of the addition of milk to tea on *in vitro* antioxidant potential were investigated.

Materials And Methods

The antioxidant power of 29 types of tea was determined by the FRAP assay (Benzie & Strain, 1996b; Benzie & Strain, 1999; for details and automated programme used see Appendices 1 & 2) The 29 types comprised 2 herbal teas, 5 Oolong teas, 9 black teas and 13 green teas. A 5% (w/v) infusion of each tea was

prepared on the day of testing by infusing 1 gram of dried tea leaves with 20 ml boiling distilled water for 10 min. The infusion was cooled and filtered and further diluted in distilled water as necessary (generally 1 in 20 dilutions to give 0.25%). The FRAP assay was performed in quadruplicate for each infusion in different runs by using a COBAS FARA centrifugal analyzer (Roche Diagnostics Ltd., Basel, Switzerland). Each type of freshly prepared tea infusion was assayed 3 times on different days. The infusions were assumed to contain all soluble antioxidants of the dried tea leaves. The 12 results of each tea were expressed mean (SD) in μmol antioxidant power (as FRAP) per gram of dried tea leaves, as $\mu\text{mol/l}$ of a 5% infusion, and as μmol antioxidant power in a typical serving of tea as usually drunk (200 ml of 1 % (w/v) tea infusion). The antioxidant power of freshly prepared, aqueous solutions of ascorbic acid (d-alpha ascorbic acid, extra pure crystals; Merck, Germany) were also measured, and the calculated antioxidant power of 1 g of pure ascorbic is given as reference.

For the stability study of antioxidant power in tea infusions, Twenty-seven types of tea infusion were kept at 4°C for 48 hours. The infusions were then re-assayed in duplicate and the results compared to those obtained on fresh samples.

For the relationship between antioxidant power and levels of tea polyphenols, FRAP values and the total phenolics content of infusions of 26 different teas were compared in a separate experiment (not all tea were included due to insufficient sample). Total phenolics content was measured by a manual Folin-Ciocalteu method (Theis & Benedict, 1924; Das, 1971). In this method, the reduction of heteropolyphosphotungstate-molybdate complex by polyphenol will

lead to a blue coloured product which can be measured by a change in absorbance at 660 nm (Singleton *et al.*, 1999) (For detailed procedure of polyphenol measurement, see Appendix 3). Precision of total phenolic content measurement was good, between-run CVs were 7% at 2.3 mmol/l, 6% at 9.2 mmol/l and 4% at 13.8 mmol/l. The linearity is excellent up to 16.8 mmol/l ($r=0.999$, $p<0.0001$).

For the *in vitro* effect of milk on tea antioxidant power, tea infusions of one selected black, oolong and green tea were prepared as previously described. The infusions were then diluted with skimmed milk. The mixture were further diluted in water (1 in 20) and the resultant mixture were then tested by FRAP for in triplicate times on the same day as preparation. The FRAP values of each tea with and without milk were compared. A blank solution of skimmed milk in water was also tested.

Results

Different types of tea had widely different antioxidant powers; ranging from 92 to 1144 $\mu\text{mol/g}$. Consolidated results are presented in Table 5.1. Each gram of herbal (Lubouma and Lingzhi) tea leaves contained 109 μmol FRAP while the average value of oolong tea was 373 μmol , of black tea was 337 μmol and of green tea was 656 μmol . Therefore, in a typical cup of tea with 200 ml volume and 1% strength, the antioxidant intake ranges from 184 μmol for herbal tea ('Luobuma tea') to 2289 μmol for green tea ('China green tea'). There was a linear relationship between the strength (w/v) of the tea infusion and the FRAP value (Figure 5.2) over a wide range of values, with close agreement between expected and observed FRAP values ($r=0.998$, $p<0.0001$). There was no

significant change ($p=0.9712$) observed in the FRAP values after 48 hours storage of tea solution at 4°C (paired t-test), indicating that the antioxidants in infusions of tea are stable (Figure 5.1).

There was a strong correlation between the FRAP value and total phenolics content of the tea infusions tested ($r=0.956$, $p<0.0001$), indicating that the number of phenolic hydroxyl groups is a major determinant of antioxidant power of tea (Figure 5.3). A non-zero intercept was obtained indicating other non-phenolic constituents possessing antioxidant power might be present, e.g. ascorbic acid. Intercept at 2889 $\mu\text{mol/l}$ indicates very small contribution of non-phenolic antioxidants to the total *in vitro* antioxidant power.

Good agreement between the expected FRAP value of the milk and tea mixture and the observed values were obtained, indicating milk showed no *in vitro* effect on the tea antioxidants (Figures 5.4, 5.5, 5.6).

Discussion

Tea, particularly green tea, is a potentially rich dietary source of antioxidant power. Various studies have demonstrated *in vitro* radical trapping antioxidant properties of black tea and green tea, of extracts of tea, and of individual polyphenolic compounds found in tea (Zhao *et al.*, 1989; Scott *et al.*, 1993; Lin *et al.*, 1996; Paganga *et al.*, 1996; Rice-Evans *et al.*, 1996; Zhang & Shen, 1997). This current study presents new data comparing the *in vitro* antioxidant / reducing power of different types of teas, as they would be drunk, using an objective and highly reproducible measure, the FRAP assay for antioxidant / reducing power. It

is worth noting that, while tea polyphenolics are known to bind metal ions (Miller *et al.*, 1996; Paganga *et al.*, 1996; Van Acker *et al.*, 1996), this property does not interfere with the measurement of antioxidant power of tea using the FRAP assay because Fe^{3+} ion in the FRAP reagent is present in excess. This was demonstrated by a linear response when the antioxidant power was tested over a wide range of concentrations of tea. In addition, when the FRAP assay was run with no Fe^{3+} in the test reagent, no colour developed with samples of tea, indicating that tea did not contain Fe^{2+} .

Binding of metal ions, such as iron, *in vivo* is an antioxidant action of itself, preventing metal ion catalysed generation of reactive species (Emerit, 1994; Halliwell, 1996). However, tea polyphenolics also have electron donating antioxidant properties, the relative activity of the different polyphenolic compounds being related to the number and location of the hydroxyl groups and the presence of the galloyl moiety (Cook & Samman, 1996; Lin *et al.*, 1996; Miller *et al.*, 1996; Paganga *et al.*, 1996; Rice-Evans *et al.*, 1996). During the process referred to as fermentation, flavanols and flavonols in green tea leaves, mainly catechins and their gallic esters, undergo a polyphenol oxidase catalysed oxidative polymerisation which turns the leaves black (Graham, 1992; Weisburger, 1996; Nanjo *et al.*, 1996). During several hours, much of the catechin content of green tea is converted to the polyphenolic condensation polymers thearubingens and theaflavins, which give black tea its characteristic astringency. Oolong tea is intermediate in composition between green and black tea as fermentation is stopped after a short time. Almost 80% of the tea consumed throughout the world

each year is black, less than 2% is Oolong, and 20% green tea. (Balentine, 1992; Kong, 1993).

The effect of milk on bioavailability of tea antioxidants was contradictory. One group showed that milk can inhibit the absorption of tea antioxidant (Serafini *et al.*, 1996) while the other group showed no effect on the absorption of catechins from tea (van het Hof *et al.*, 1998). In this part of the study, at least no effect of milk on *in vitro* tea antioxidants was demonstrated.

This part of study has confirmed that the antioxidant power of green tea is considerably higher than black tea, while Oolong tea, expected to be intermediate, appeared similar to black tea. Herbal tea had lowest antioxidant power. The herbal tea tested was from the plant of Luobuma (*Apocynum venetum*) and Lingzhi (*Ganoderma lucidum*) rather than the traditional tea plant (*Camellia sinesis*). Values overlapped, however, with a two to three-fold difference across different brands of teas of the same type, probably reflecting differences in quality, geographical regions of growth, the time of year when the leaves were picked, and varying storage conditions (Lin *et al.*, 1996; Weisburger, 1996). Nevertheless, the potential for all types of tea (including herbal teas) to contribute significantly to the dietary intake of antioxidant power is high. The recommended daily allowance (RDA) of vitamin C is about 40 - 60 mg (UK and US standards respectively)(equivalent to 453-680 μmol antioxidant power as FRAP). One or two cups of green tea (containing 1300-2600 μmol FRAP) can provide 2-6 times the antioxidant power of the RDA ascorbic acid. Vitamin C is an antioxidant of established importance, while the bioavailability and role of tea antioxidants are

not yet clear. Nonetheless, tea may be an important dietary source of antioxidant power and tea can be ingested frequently and in relatively large volumes without any known harmful effects. It should be noted, however, that tea can inhibit non-haem iron absorption (Kaltwasser *et al.*, 1998). And although concern was made to the high oxalate content in tea increasing the risk of kidney stones (Finch *et al.*, 1981), the low bioavailability of oxalate in tea opposed this view (Brinkley *et al.*, 1981; Brinkley *et al.*, 1990). However, further investigation on the gastrointestinal absorption and bioavailability of antioxidants in tea is required to confirm if the obvious potential of tea can be translated into a real and useful dietary source of antioxidant power. Bioavailability studies to date are conflicting with reference to whether or not antioxidant power of tea is absorbed. Furthermore, the effect of the addition of milk to tea and its antioxidant potential is disputed (Serafini *et al.*, 1996; van het Hof *et al.*, 1998). In this current study no effect on *in vitro* antioxidant power was seen when milk was added to green, oolong or black tea.

In summary, this part of the study has shown that green teas have the highest antioxidant power and black teas have lowest values. Oolong teas, expected to be intermediate between green and black teas, were similar to black teas. Also, antioxidants in tea were found to be stable for at least 48 hours stored at 4°C and addition of milk did not change the antioxidant content of tea. Further study into the absorption and effect of tea antioxidants on antioxidant status is needed to evaluate their potential benefit in the maintenance of human health.

Data and information presented in this chapter have been published in:

Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay.

Journal of Agricultural and Food Chemistry. 1999, 47 (2), 633-636. (with IFF Benzie)

Table 5.1 Summary of antioxidant activities in dried tea leaves or 1 cup of tea (200 ml 1% infusion) (mean of 3 different infusions)

Tea Sample	Name	Type	FRAP value of tea leaves, mean + SD ($\mu\text{mol/g}$ dried leaves)	Estimated FRAP value in 1 cup of tea, mean + SD (μmol)
B1	China Black Tea	Black	379 (68)	757 (136)
B3	Rose Black Tea	Black	378 (28)	756 (56)
B4	Yunnan Tuocha	Black	358 (57)	716 (114)
B5	Breakfast Tea (extra strong)	Black	654 (73)	1278 (146)
B6	Pu Li Tea	Black	309 (58)	619 (116)
B7	Pu Li Tea	Black	225 (18)	450 (36)
B8	Pu Li Tea	Black	238 (40)	477 (80)
B9	Pu Li Tea	Black	380 (27)	760 (54)
B10	Premium Yun-Nan Puerh	Black	132 (16)	264 (80)
G1	China Green Tea	Green	1144 (10)	2289 (20)
G2	Jasmine Tea	Green	629 (95)	1258 (190)
G3	Jasmine Tea	Green	526 (50)	1052 (100)
G4	Jasmine Tea	Green	602 (69)	1275 (138)
G5	Jasmine Tea	Green	698 (96)	1395 (192)
G6	Lok On (Ngan Jum)	Green	727 (82)	1454 (164)
G7	Lok On (Ngan Jum)	Green	525 (27)	1050 (54)
G8	Shui Sin Tea	Green	522 (77)	1045 (154)
G9	Shui Sin Tea	Green	272 (7)	544 (14)
G10	Shou Mei Tea	Green	386 (34)	771 (68)
G11	Yunnan Green Tea	Green	996 (118)	1991 (236)
G12	Japanese Green Tea	Green	700 (95)	1401 (190)
G13	China Green Tea	Green	699 (134)	1398 (268)
O1	Oolong Tea	Oolong	532 (33)	1063 (66)
O2	Oolong Tea	Oolong	233 (31)	466 (62)
O3	Iron Buddha (Tikuanyin Tea)	Oolong	472 (45)	944 (90)
O4	Iron Buddha (Tikuanyin Tea)	Oolong	340 (42)	680 (84)
O5	Iron Buddha (Tikuanyin Tea)	Oolong	289 (50)	578 (100)
L1	Lingzhi Tea	Herbal	126 (8)	251 (16)
L2	Luobuma Tea	Herbal	92 (19)	184 (38)

* Ascorbic acid: 11364 μmol FRAP per gram

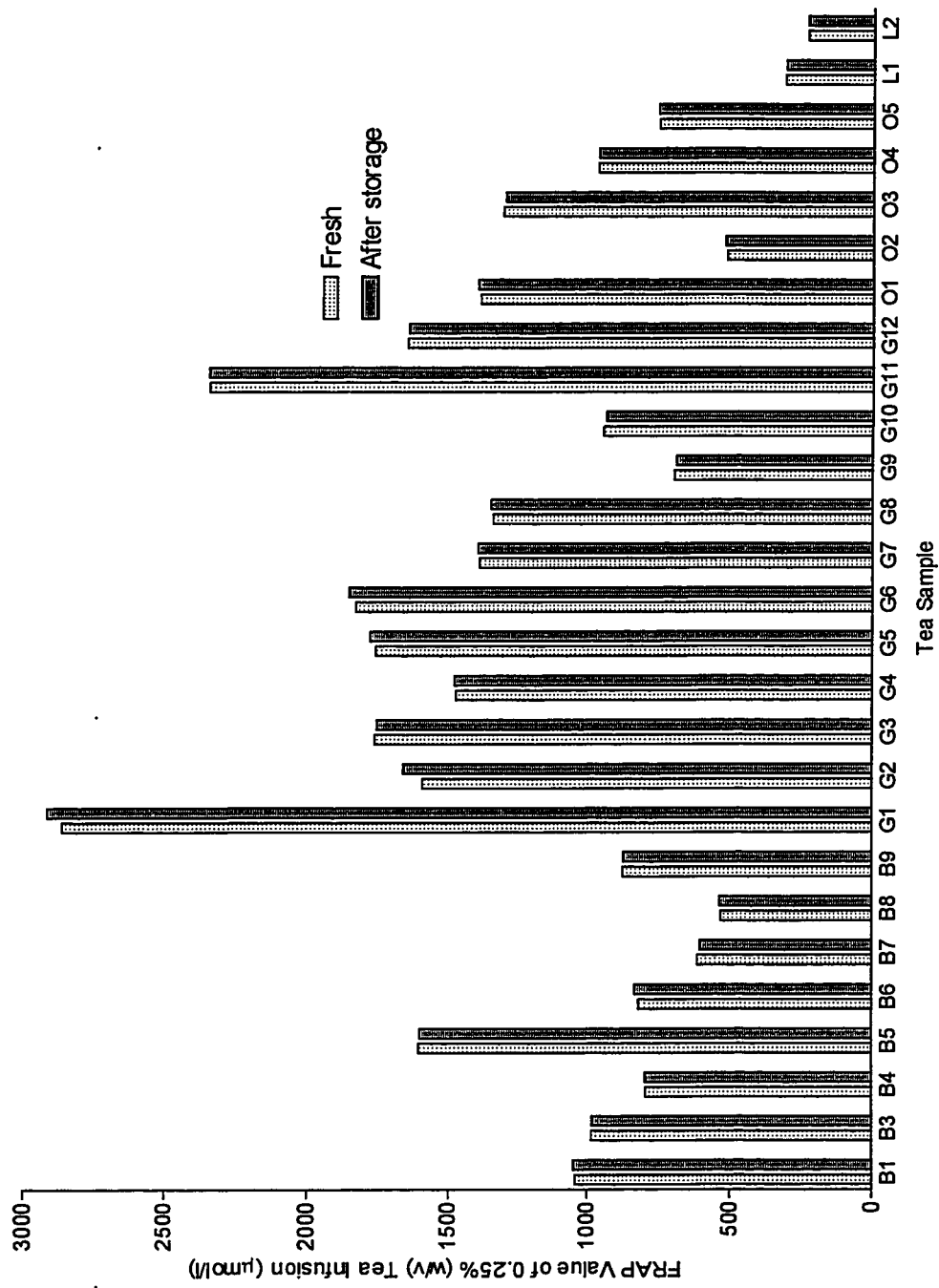


Figure 5.1 Change of FRAP value of tea infusions after 48 hours storage at 4°C. Code for teas as in Table 5.1.

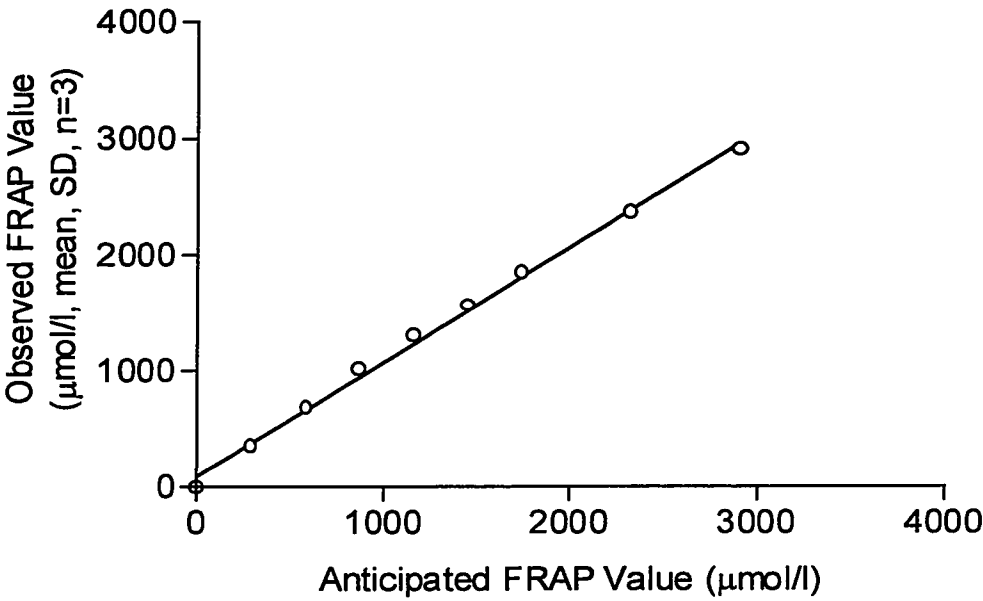


Figure 5.2 FRAP values of infusion of a selected green tea at different dilutions. Result show good agreement ($r=0.998$, 95% CI=0.993 to 1.000 $p<0.0001$) between the anticipated and the observed (measured) FRAP value, indicating no change in relative antioxidant power at different concentrations. Each point represents the mean of triplicate measurements, and while 1 SD error bars are included, reproducibility was high and error bars do not show.

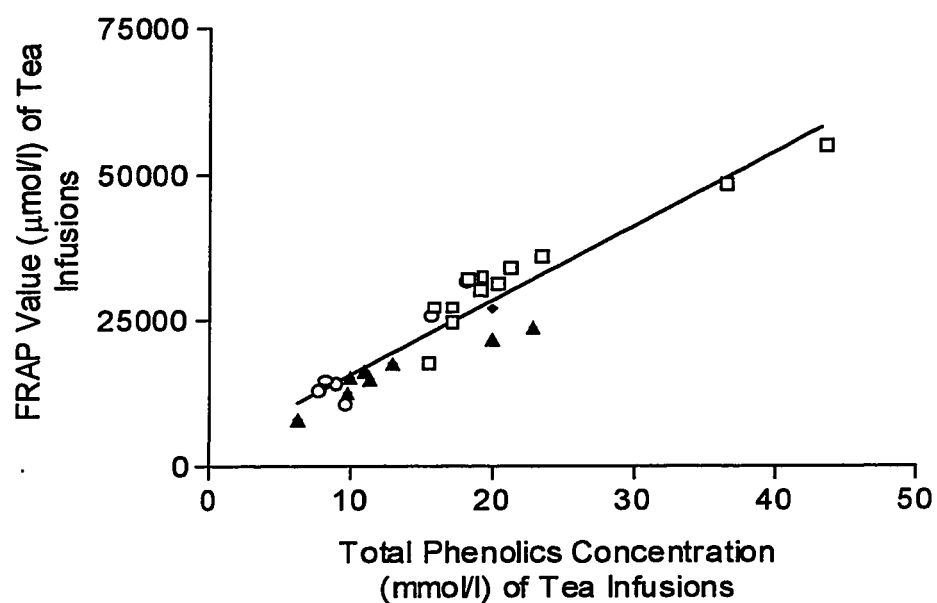


Figure 5.3 FRAP values and total phenolics content of 26 different tea infusions of 5% (w/v) strength. Green tea infusions are represented by squares, oolong tea by circles and black tea by triangles. Results showed strong correlation ($r=0.953$, 95% CI=0.896 to 0.979, $p<0.0001$) between the two parameters. Each point represents the mean of two individual experiments, each experiment with duplicate measurement, i.e. mean of 4 results of each tea.

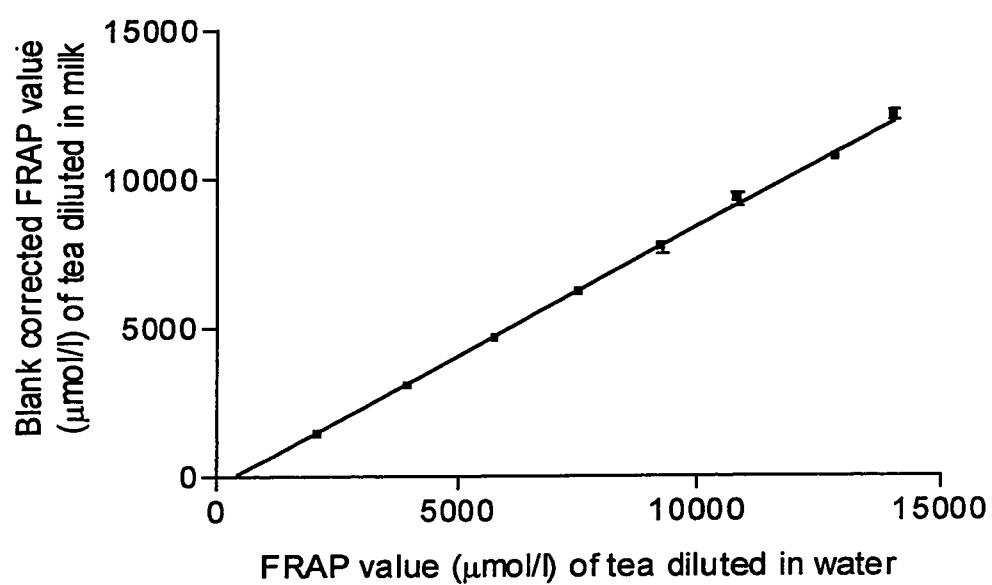


Figure 5.4 Effect of milk on black tea antioxidant power at different dilution. Each point represents triplicate measurements of the specimen and 1 SD error bars.

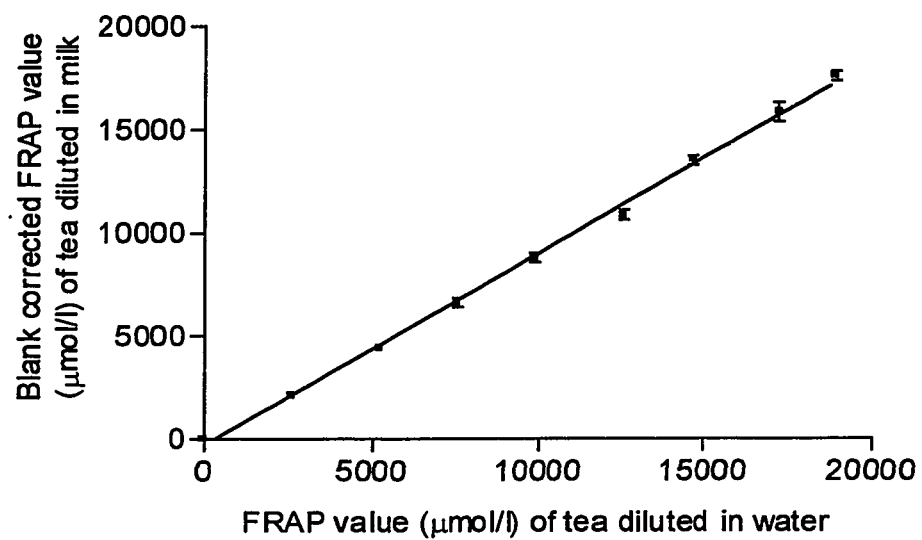


Figure 5.5 Effect of milk on oolong tea antioxidant power at different dilution. Each point represents triplicate measurements of the specimen and 1 SD error bars.

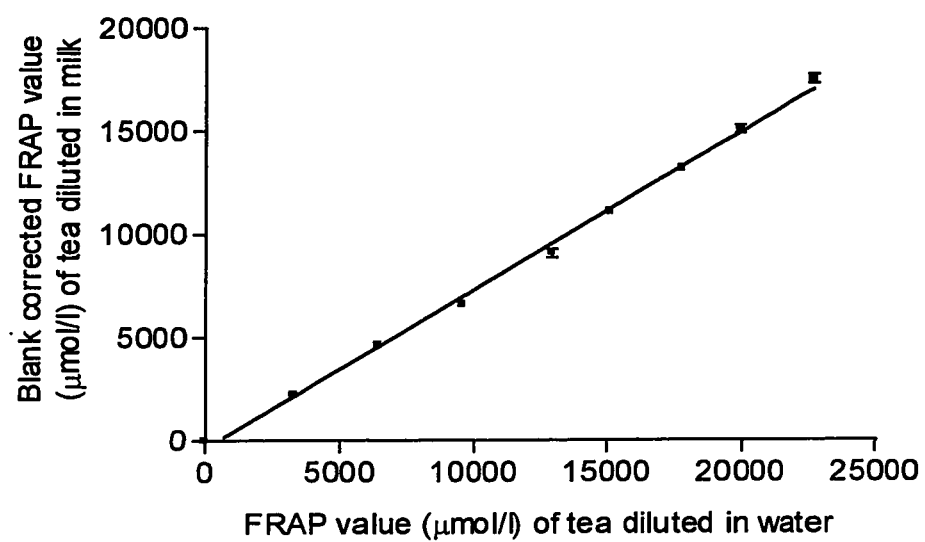


Figure 5.6 Effect of milk on green tea antioxidant power at different dilution. Each point represents triplicate measurements of the specimen and 1 SD error bars.

CHAPTER 6 ABSORPTION OF GREEN TEA ANTIOXIDANTS

Introduction

Animal studies have shown that tea antioxidants can protect against powerful mutagens (Yamane *et al.*, 1991; Klaunig 1992; Mukhtar *et al.*, 1992; Stich, 1992; Hasegawa *et al.*, 1995; Nanjo *et al.*, 1996; Lin *et al.*, 1996; Leanderson *et al.*, 1997), and some epidemiological studies have reported a lower incidence of cancer in association with high intake of tea (La Vecchia *et al.*, 1992; Baron *et al.*, 1994; Gao *et al.*, 1994; Yu *et al.*, 1995). An inverse association was observed between drinking green tea and coronary heart disease (CHD), and with serum markers of CHD risk such as total cholesterol and LDL-cholesterol (Klatsky *et al.*, 1993; Imai & Nakachi, 1995). A clearly beneficial health effect role of tea antioxidants, however, has not been established to date (Kinlen *et al.*, 1988; Goldbohm *et al.*, 1996; Kohlmeier, 1997; Kohlmeier *et al.*, 1997; Wiseman *et al.*, 1997).

However powerful the *in vitro* antioxidant and/or anticarcinogenic activity of tea antioxidants may be, potential health benefits are unlikely to be realised if tea antioxidants are inactivated in the gut or are not absorbed. Absorption studies in human subjects to date are few, and show conflicting results (Das, 1971; Gugler, 1975; Hollman *et al.*, 1996 van het Hof *et al.*, 1998). In studies assessing absorption and systemic distribution of tea antioxidants, post-ingestion response in the 'total antioxidant power' of plasma can be used as a marker of absorption, the mechanism being that if antioxidants are absorbed with their activity

conserved, the antioxidant power of plasma will increase. Results of the two small tea antioxidant studies of this type published to date (Maxwell & Thorpe, 1996; Serafini *et al.*, 1996), however, show no agreement: no detectable post-ingestion change in the total antioxidant power of plasma was reported by one group (Maxwell & Thorpe, 1996), while the other (Serafini *et al.*, 1996) reported average increases of 40-50%, and approaching 100% in some subjects, within 1 hour. These studies used different methods of assessing total antioxidant power, and a combination of methodological difference, insufficient dosing with tea antioxidants (500 ml of 1 % black tea infusion, in the study of Maxwell & Thorpe), different sampling times, such as 30-60 min of first blood sample after ingestion, and possible effects of storage on antioxidants in plasma prior to analysis (Benzie & Chung, 1999) may help account for the conflicting results. Methodological differences notwithstanding, however, it is difficult to reconcile a near doubling of plasma antioxidant power in one study with an undetectable response in the other. It is not yet clear, therefore, whether antioxidant compounds in green or black tea are absorbed in significant amounts following ingestion. Further study into the bioavailability and potential beneficial effects of tea antioxidants is clearly needed.

Aims

The aims of this part of the study were to investigate the absorption in humans, of the tea antioxidants. In these experiment, changes in antioxidant power were monitored, in both plasma and urine, at timed intervals after ingestion of a known amount of green tea. The marker of antioxidant absorption was the FRAP value (Benzie & Strain, 1996b; Benzie & Strain, 1999) which enables sensitive and reproducible results to be obtained on complex biological samples, and which

is simple and rapid enough to be performed on freshly collected specimens. Owing to its speed and high sensitivity, exactly timed, serial samples can be measured and small post-ingestion differences can be detected. This makes the FRAP assay a useful bioavailability tool.

In addition to monitoring post-ingestion changes in plasma and urinary antioxidant power, urinary excretion of total phenolics was also measured. This was correlated with urinary excretion of antioxidant power following ingestion of green tea in order to evaluate the probable cause of antioxidant power changes in urine.

Materials And Methods

Subjects: a total of 12 healthy adults consented to take part in the study, however, owing to difficulty in obtaining serial blood samples from one subject and gastric intolerance of the tea preparation in another, results are presented for 10 (5 male, 5 female) subjects. This study was approved by the Ethics Subcommittee of the Hong Kong Polytechnic University (Appendix 4) and all procedures involving human subjects complied with the declaration of Helsinki, as revised in 1996.

Test methods: the FRAP assay was performed, as described in Chapter 3. Total phenolics in urine were measured using the Folin-Ciocalteu reaction as described in Chapter 5 (Detail procedure, see Appendix 3). Creatinine was measured in plasma and urine using an alkaline picrate test kit (Roche) on a Cobas Fara. FRAP assay results were expressed as $\mu\text{mol/l}$ antioxidant power for plasma,

and as μmol antioxidant power per μmol creatinine for urine. Total phenolics (μmol) in urine were also reported per μmol creatinine.

Preparation of tea: strong green tea (5% w/v) was freshly prepared on each occasion by adding 500 ml boiling distilled water to 20 g dry tea leaves ('China green tea', Shanghai Tea Import and Export Corporation) purchased from a local shop; the tea was allowed to infuse for 10 minutes, filtered, and the final volume adjusted to 400 ml with distilled water. The FRAP value of small aliquot of each tea infusion, as drunk, was measured within 3 hours of preparation. Green tea was chosen because a strong dosage of antioxidants (see Chapter 5) was desirable in order assess changes in both plasma and urine antioxidant power.

Protocol and samples: Subjects (a maximum of 3 on any morning) were asked to arrive fasting. Informed consent was obtained, after which a heparinised blood sample and a urine sample were collected from each subject; 400 ml – or as much as each subject could comfortably drink, which in two of the ten subjects was 260 and 290 ml only – of tea was ingested over the next 10 minutes. Additional venous blood samples were collected into heparinised blood collection tubes at 20, 40, 60 and 120 minutes post-ingestion. Blood samples were kept chilled and in the dark until separation of plasma from the erythrocytes, which was within 2.5 hours of blood collection. The plasma total antioxidant/reducing power, as FRAP, was measured in triplicate immediately thereafter. Urine samples were collected, at 30 minute intervals, without preservative into clean glass containers for up to 3 hours post-ingestion. The urine FRAP values and phenolics content were measured, after appropriate dilution in distilled water,

within 4 hours collection. Subjects remained fasting, except for the initial ingestion of tea and subsequent sips of distilled water, for the entire period of sample collection. At least 4 weeks later seven of the ten subjects (five male and two female) repeated the study, drinking warm distilled water in place of tea (Schematic test procedure, see Figure 6.1).

It should be noted that, while it was planned that all volunteers would take the 400 ml of 5 % tea, giving a similar dose of antioxidants in each case, 2 volunteers found the strong, bitter tea distasteful, and drank around 300 ml only; the other eight subjects drank 400 ml. The doses, were not standardised or related to body mass index, but were known: mean (SD) doses of tea and antioxidant power for the 10 subjects were 375 (53) ml and 18909 (7106) μmol respectively. Six of the 10 subjects collected all urine passed at each 30 minute interval over 3 hours; four subjects retained only aliquots at each time period.

For analysis of results, the following were calculated:

- Peak post-tea ingested increase in FRAP over fasting plasma levels
- Area under the curve (AUC) for increase in plasma FRAP over 2 hours
- Correlation of AUC with dose of antioxidants ingested
- Correlation of baseline plasma FRAP level and response, as AUC
- Increase in urinary FRAP following ingestion of tea
- Correlation between phenolics and FRAP in urine following ingestion of tea
- Amount of ingested antioxidants and antioxidants excreted over 3 hours (in 6 subjects only)

Statistical analysis: Kruskal-Wallis test was used to investigate timed post-

ingestion changes in plasma FRAP values (n=10). The Dunn's Multiple Comparison test was used to investigate timed post-ingestion changes in plasma FRAP values after ingestion of tea and water, and to compare baseline (fasting) plasma FRAP values prior to ingestion of tea and of water (n=7).

Results

Absorption of tea antioxidants from green tea was fast, resulting in a significant ($p<0.001$) increase in the FRAP value of plasma. The peak increase occurred at 20-40 minutes post ingestion, and the mean (SEM) 40 minute increase was 44 (9) μmol (Figure 6.2, Table 6.1), or around 4%. The magnitude of response was not related to either the dose of tea antioxidants ingested or to the baseline FRAP value (Table 6.2, Figures 6.3 & 6.4). The increase in plasma FRAP was of short duration, and values returned to or approached baseline (fasting) levels by 2 hours post-ingestion in most, though not all, subjects. Plasma creatinine concentrations did not change during the course of the study, indicating no confounding net changes in the plasma water content owing to ingestion of liquid. In subjects who repeated the test with water in place of tea, fasting plasma FRAP values were not significantly different from on the previous occasion [mean (SEM) 1156 (65) $\mu\text{mol/l}$ and 1169 (53) $\mu\text{mol/l}$ respectively]. No post-ingestion increases in plasma FRAP were seen after ingestion of water (Figure 6.2, Table 6.3).

The area under the 2 hour-curve (AUC_{0-2h}) was calculated (GraphPad Prism 2.01), based on increases in plasma FRAP from baseline up to 2 hours post-ingestion of tea. There was no direct correlation between dose of tea antioxidants

ingested and AUC_{0-2h} between subjects; nor was any significant correlation seen between dose and peak increase in plasma FRAP (Figures 6.4 & 6.5, Table 6.2).

The increase in urinary excretion of antioxidant power was fairly rapid, with peak excretion at 60-90 minutes post-ingestion. The pattern was similar to that of total phenolics excretion (Figures 6.6, 6.7, Tables 6.4, 6.5, 6.6, 6.7). Furthermore after tea, but not after water, there was a significant correlation between urine antioxidant power and total phenolics concentration ($r=0.845$; $p<0.001$) (Figures 6.8 & 6.9). This indicates that the increase in antioxidant power of urine was related to excretion of the polyphenolics antioxidants absorbed owing to ingestion of green tea. There was no apparent relationship between plasma response, in terms of AUC over 2 hours, and the total amount of antioxidant power excreted (as μmol FRAP) (Figure 6.10).

Discussion

Human studies of absorption of polyphenolics antioxidants in tea have, to date, been few and their results conflicting (Das, 1971; Gugler *et al.*, 1975; Kinlen *et al.*, 1988; Hollman *et al.*, 1996; Maxwell & Thorpe, 1996; Serafini *et al.*, 1996). In the current study, results demonstrated a clear and rapid 'spike' of antioxidant power into the plasma after ingestion of green tea, indicating that at least some of the antioxidants in green tea are absorbed and reach the systemic circulation. Excretion of absorbed antioxidant also appeared to be rapid, mirroring excretion of phenolic compounds in the urine.

The speed (peak at 20-40 minutes) and magnitude (average increase 4%) of

response seen in this current study could account for the apparent lack of response reported in a previous study, in which black tea was taken (Maxwell & Thorpe, 1996). Blood samples were collected between 1 and 3 hours post ingestion, thereby missing the peak tea-related increase in plasma antioxidant power seen in this current study, and the very small increases remaining 1 hour post-ingestion may well have been undetectable by the method used. And in addition the method used was a one-by-one test which required a very long time period for a large numbers of specimens. Fresh samples may not be processed immediately.

In this current study, peak responses in all subjects were similar, and plasma FRAP values approached baseline by two hours post-ingestion in most subjects. Two subjects showed a prolonged response, however, indicating that total absorption and metabolism of tea antioxidants may differ between individuals. The difference may be related to prolonged absorption and/or differential metabolism or elimination in some individuals. This could help account for the varying epidemiological findings reported to date (Goldbohm *et al.*, 1996; Blot *et al.*, 1997; Kohlmeier *et al.*, 1997). Individual, perhaps genetically modulated, response to tea consumption deserves further study. No correlation was seen between dose of tea antioxidant and plasma response in the current study, possibly indicating that the absorption mechanism was saturated at even the lowest dose of tea ingested (10345 μmol FRAP), and/or that absorption of antioxidants in tea is selective. It is interesting to note that, in a small ($n=4$) study (Lee *et al.*, 1995) of absorption of polyphenols from green tea, measured plasma levels of individual catechins (EGCG, EGC, ECG, EC) at one hour post-ingestion covered a very wide range. Furthermore, peak concentrations of catechins in plasma and urine

have been reported to occur at between 1 and 4 hours and 3 and 6 hours post-ingestion respectively (Lee *et al.*, 1995; van het Hof *et al.*, 1998; Hollman *et al.*, 1997). This implies that the rapid post-ingestion increase in antioxidant power seen in this current study, which peaked at 20-40 minutes, may have been mediated by non-catechin phenolic antioxidant(s), and this deserves further study.

Results of this study showed a mean (SEM) plasma AUC of 61.1 (15.4) $\mu\text{mol/l/2h}$ in the 2 hours after ingestion of green tea. This indicates a rather poor systemic availability of antioxidants from tea, but supports the rapid response seen in the single published study which reported a detectable post-ingestion response. The mean increase in plasma antioxidant power following one cup (300 ml) of 2 % (w/v) tea was, however, surprisingly large (45%; n=5) in that previous study (Serafini *et al.*, 1996). Such large variation is difficult to explain, but may be related to individual differences in absorption or elimination as suggested above. Nevertheless, while individual and methodological differences may have contributed to the differences in magnitude and variation of response seen, results of both the current study and the previous study (Serafini *et al.*, 1996) indicate that absorption and systemic distribution of ingested tea antioxidants are rapid.

Results presented here indicate that at least some of the antioxidants in green tea are absorbed and systemically distributed. The four percent increase in antioxidant seen is not a high response, but the uptake of antioxidants by tissue was not accounted in this study. Actual absorption of tea antioxidants may be higher than 4 %. Regular consumption of green tea could, therefore, improve antioxidant defence and lower risk of disease associated with increased of

oxidative stress. Furthermore, tea antioxidants left unabsorbed in the gut may act to conserve other dietary antioxidants, and could confer local protection before excretion or microbial degradation. Whether or not absorption of tea antioxidants is of significant benefit to health remains to be established, however. Intake of black, as opposed to green, tea is high throughout the United Kingdom and Ireland, but cancer and CHD have clearly not been eradicated in these areas. Epidemiological studies, however, have no power to discriminate or reveal differences if rates of disease and/or the population means of the agent under investigation do not vary significantly between the populations being studied (Benzie, 1998). In addition, variables such as socio-economic status, smoking and dietary habits combine to confound results, and dose-related effects of black tea have yet to emerge (Blot *et al.*, 1997; Hertog *et al.*, 1997). It has been suggested that taking tea with milk, which is the preferred way in which black tea is taken in UK and Ireland, may affect absorption and/or antioxidant action of tea antioxidants (Serafini *et al.*, 1996; Hertog *et al.*, 1997). It is interesting to note, however, that milk does not appear to affect the absorption of catechins (van het Hof *et al.*, 1998) or the *in vitro* antioxidant power (Chapter 5). Further study of factors affecting absorption of tea antioxidants, including characterisation of the individual antioxidant(s) responsible for the rapid post-ingestion increase in plasma antioxidant power seen in this study, is clearly needed.

In summary, results of this part of the study confirm that polyphenolic antioxidant compounds in green tea are absorbed and enter the systemic circulation rapidly after ingestion, and that their absorption causes a small but significant increase in plasma antioxidant power. Further study is needed to

establish whether regular ingestion of green tea leads to a significant response in terms of decreased oxidative damage to DNA and lipid and, thereby, to lowered cancer and cardiovascular risk in humans, to investigate which antioxidant(s) in green tea are bioavailable, and to investigate if some individuals are more responsive to green tea antioxidants than others.

Data presented in this chapter have been accepted for publication in:

Consumption of green tea causes rapid increase in plasma antioxidant power in humans. *Nutrition and Cancer – An International Journal*. 1999, 34 (1), 83-87.

(with IFF Benzie, JJ Strain, B Tomlinson)

Table 6.1 Increase in plasma antioxidant power after ingestion of green tea

Subject	FRAP Value ($\mu\text{mol/l}$) of Plasma Different Time [(Mean of triplicate (SD))]			
	20 min	40 min	60 min	120 min
A	49 (5)	26 (9)	17 (4)	6 (7)
B	21 (6)	30 (7)	21 (3)	7 (0)
C	22 (12)	31 (15)	20 (16)	-6 (14)
D	22 (31)	26 (21)	24 (26)	10 (25)
E	65 (11)	98 (2)	*	100 (4)
F	*	*	54 (26)	31 (9)
G	*	33 (11)	25 (5)	-1 (8)
H	67 (8)	42 (15)	30 (12)	29 (18)
I	25 (11)	63 (12)	*	68 (5)
J	18 (21)	*	30 (19)	2 (17)

*= results excluded because samples haemolysed

Table 6.2 Baseline plasma FRAP, dose of antioxidants ingested and individual responses after ingestion of green tea

Subject	Baseline Plasma FRAP ($\mu\text{mol/l}$)	Dose of FRAP ingested (μmol)	Plasma FRAP changes, $\text{AUC}_{0-2\text{h}}$ ($\mu\text{mol/l.2h}$)	FRAP excreted (μmol)	Peak Change of Plasma FRAP ($\mu\text{mol/l}$)
A	1123	18600	13.59	n.d.	49
B	926	18600	18.05	n.d.	30
C	1445	36600	13.03	n.d.	31
D	1279	25000	18.67	2384	26
E	1210	16000	85.00	2815	100
F	932	15200	34.75	n.d.	54
G	1177	10300	16.34	3271	33
H	1149	15900	35.42	1913	67
I	995	20900	53.08	2376	68
J	1167	20900	17.50	2297	30

n.d. = not done

Table 6.3 Changes in plasma antioxidant power after ingestion of water

Subject	Increase in FRAP Value ($\mu\text{mol/l}$) of Plasma at Different Time after ingestion of water [(Mean of triplicate (SD))]			
	20 min	40 min	60 min	120 min
A	-1 (4)	-20 (6)	-25 (3)	-14 (6)
B	2 (3)	-7 (4)	-3 (9)	*
C	-15 (20)	-23 (8)	-5 (4)	-12 (2)
D	-6 (11)	-5 (6)	-34 (7)	-51 (14)
E	n.d.	n.d.	n.d.	n.d.
F	n.d.	n.d.	n.d.	n.d.
G	-15 (11)	*	0 (15)	20 (11)
H	-12 (10)	-32 (11)	-26 (8)	-56 (5)
I	-15 (17)	-9 (5)	0 (5)	20 (11)
J	n.d.	n.d.	n.d.	n.d.

*= results excluded because sample haemolysed

n.d.= not done

Table 6.4 Antioxidant power of urine after ingestion of green tea

Subject	μmol Antioxidant Power per μmol Creatinine (mean of duplicate or triplicate readings)							
	Fasting	30 min	60 min	90 min	120 min	150 min	180 min	240 min
A	0.715	0.802	0.969	0.938	0.897	0.907	-	0.826
B	1.326	1.460	1.504	1.493	1.514	1.444	-	1.224
C	0.992	1.255	1.478	1.571	1.722	1.705	1.647	-
D	0.762	0.882	0.845	0.875	0.818	0.792	0.846	-
E	1.176	1.246	1.741	1.613	1.698	1.632	1.612	-
F	1.057	1.235	1.335	1.322	1.311	1.195	1.075	-
G	1.995	1.869	2.393	1.773	1.648	1.547	1.766	-
H	0.518	0.702	0.822	0.781	0.777	0.867	0.874	-
I	1.012	1.164	1.311	1.566	1.563	*	1.642	-
J	0.868	1.085	1.157	1.164	1.281	1.210	1.187	-

*=no urine produced

-=not done

Table 6.5 Total phenolics of urine after ingestion of green tea

Subject	μmol Total Phenolics per μmol Creatinine							
	Fasting	30 min	60 min	90 min	120 min	150 min	180 min	240 min
A	0.37	0.33	0.58	0.54	0.50	0.34	-	0.44
B	0.60	0.76	0.83	0.85	1.07	0.74	-	0.69
C	0.76	0.84	0.95	1.03	1.13	1.06	1.03	-
D	0.42	0.55	0.58	0.53	0.56	0.55	0.63	-
E	0.31	0.44	0.94	0.97	0.91	0.88	0.73	-
F	0.31	0.67	**	0.93	0.75	0.71	0.66	-
G	1.18	1.16	1.34	1.02	0.84	0.68	1.02	-
H	1.42	0.50	0.58	0.56	0.59	0.66	0.75	-
I	0.55	0.62	0.74	0.86	0.90	*	1.01	-
J	0.48	0.48	0.55	0.63	0.65	0.70	0.66	-

* = no urine produced

** = result not available

- = not done

Table 6.6 Antioxidant power of urine after ingestion of water

Subject	μmol Antioxidant Power per μmol Creatinine (mean of duplicate)						
	Fasting	30 min	60 min	90 min	120 min	150 min	180 min
A	1.18	1.21	1.11	1.06	1.02	0.99	1.00
B	0.99	0.89	0.92	0.91	0.86	0.84	0.75
C	1.12	1.36	1.41	1.40	1.14	1.25	1.20
D	0.74	0.88	0.92	0.97	0.82	0.78	0.71
E	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
F	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
G	0.90	1.12	1.40	1.13	1.07	1.12	1.16
H	0.91	0.96	0.99	0.99	0.91	0.90	0.94
I	0.94	1.08	1.00	1.14	1.10	1.14	1.03
J	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not done

Table 6.7 Total phenolics of urine after ingestion of water

Subject	mmol Total Phenolics per mmol Creatinine						
	Fasting	30 min	60 min	90 min	120 min	150 min	180 min
A	0.78	0.81	0.84	0.85	0.71	0.67	0.67
B	0.74	0.88	0.69	0.69	0.72	0.68	0.64
C	0.64	0.69	0.70	0.70	0.62	0.64	0.61
D	0.50	0.63	0.65	0.67	0.36	0.57	0.54
E	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
F	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
G	0.50	0.64	0.73	0.57	0.61	0.56	0.57
H	0.48	0.63	0.60	0.61	0.58	0.55	0.54
I	0.49	0.53	0.44	0.55	0.39	0.46	0.53
J	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not done

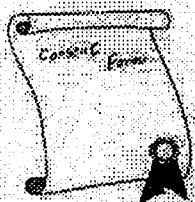
Test Procedure for Tea Antioxidant Absorption Study

Procedure of preparing tea infusion



- About 500 ml boiling distilled water is added to 20 g dried tea leaves
- Let the infusion stand for 10 min
- Filter
- Make up the filtrate to 400 ml with boiling distilled water

Procedure of tea bioavailability study



- Subject arrives fasting
- Signs the Consent Form after reading Information Sheet
- Gives personal details (name, sex, age)



- 5 ml blood is taken into heparin bottle; urine is collected in clean container; bladder should be emptied
- Blood collected should be protected from light and kept at 4°C
- Urine volume is noted and aliquot kept in clean bottle at room temperature (no preservative)

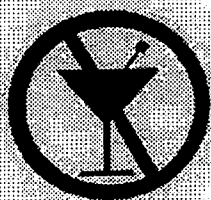
Figure 6.1 Schematic test procedure of tea antioxidants absorption study



- 400 ml strong tea (about 5%) is drunk within 10 min
- note time



- 5 ml blood is taken into heparin bottle at 20, 40, 60 & 120 min after tea is drunk; blood samples kept in dark at 4°C
- Urine is collected in a clean container at 30, 60, 90, 120, 150 & 180 min after tea is drunk; bladder should be emptied each time, volume noted and aliquot stored at room temperature



- No other food or drink (except sips of distilled water) allowed during test period (3 hours)



- Blood collected should be protected from light and kept at 4°C until separation (3000 rpm, 15 min, 4°C)
- Note the condition of plasma (e.g. haemolysis*)
- Measure FRAP (triplicate) & creatinine in plasma in all (non-haemolysed) plasma samples

- Measure total phenolics in all urine samples
- Measure FRAP (duplicate) & creatinine after appropriate dilution in distilled water (e.g. 1 in 10)
- Measure total phenolics & FRAP after appropriate dilution

* haemolysed samples should not be used

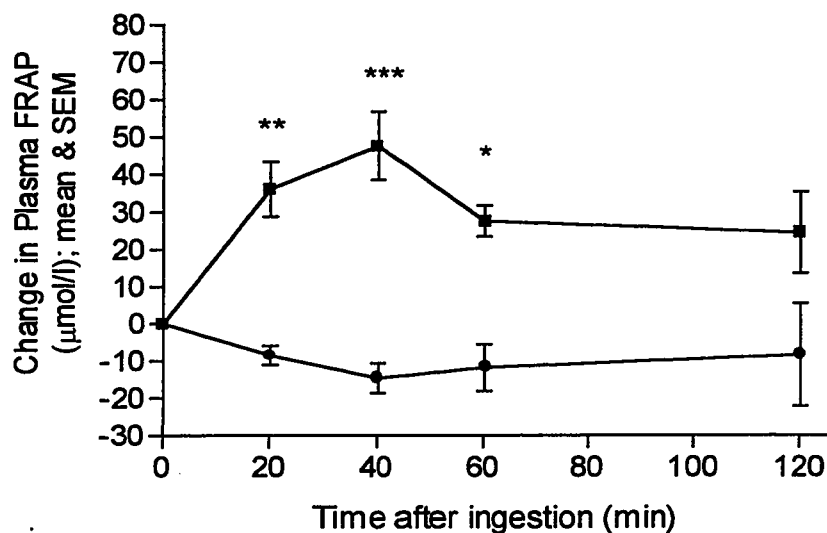


Figure 6.2 Change (mean \pm SEM, $\mu\text{mol/l}$) in plasma antioxidant/reducing power, as FRAP, after drinking green tea (squares; $n=10$) or water (circles; $n=7$). There was a rapid and significant response to tea, peaking at 40 minutes post-ingestion (significantly different from baseline (fasting) value; * $p<0.05$; ** $p<0.01$; ***: $p<0.001$ by Dunn's multiple comparison test after Kruskal-Wallis test). No significant changes in plasma FRAP values were seen after ingestion of water (Kruskal-Wallis test, $p=0.067$).

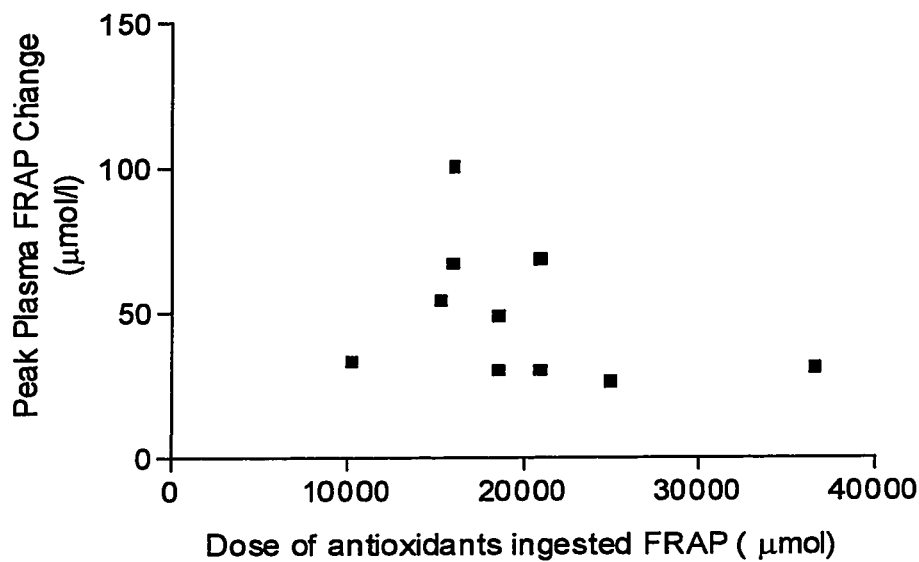


Figure 6.3 Correlation between dose of antioxidants ingested (μmol FRAP) and peak plasma FRAP change ($\mu\text{mol/l}$ FRAP) after ingestion of green tea ($n=10$). No association was found between these 2 parameters ($r=-0.349$, 95% CI=-0.802 to 0.360, $p=0.323$).

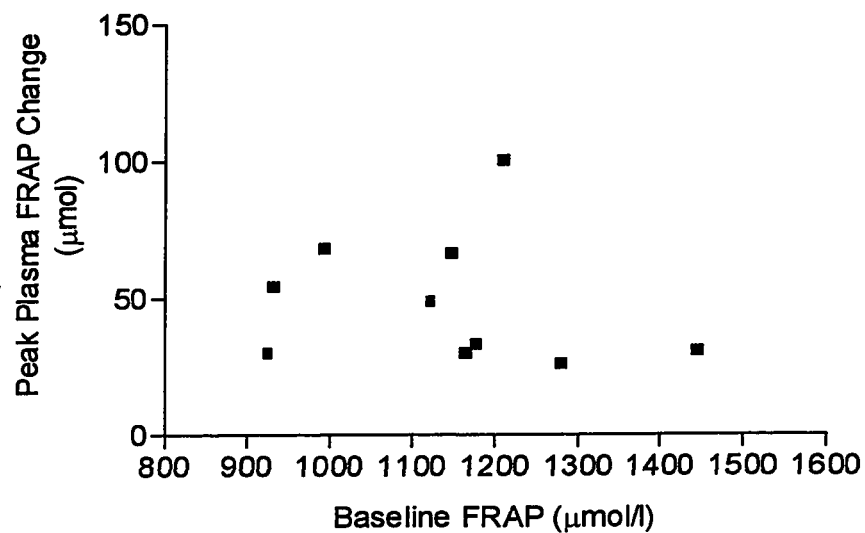


Figure 6.4 Correlation between baseline plasma antioxidant level ($\mu\text{mol/l}$ FRAP) and peak plasma FRAP change ($\mu\text{mol/l}$ FRAP) after ingestion of green tea ($n=10$). No association was found between these 2 parameters ($r=-0.168$, 95% CI=-0.746 to 0.516, $p=0.642$).

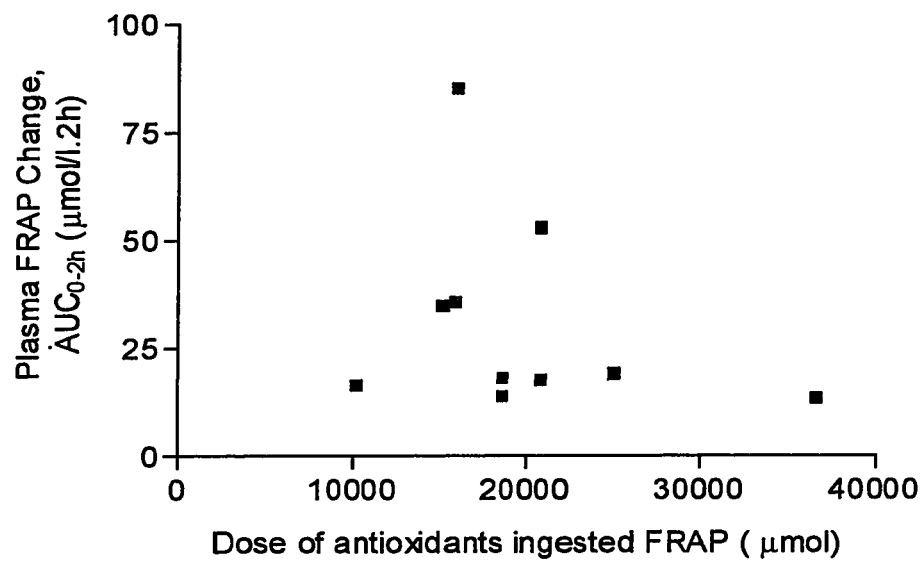


Figure 6.5 Correlation between dose of antioxidants ingested (μmol FRAP) and plasma FRAP change (Area Under the Curve, AUC_{0-2h}, μmol/l.2h FRAP) after ingestion of green tea (n=10). No association was found between these 2 parameters ($r=-0.285$, 95% CI=-0.776 to 0.420, $p=0.425$).

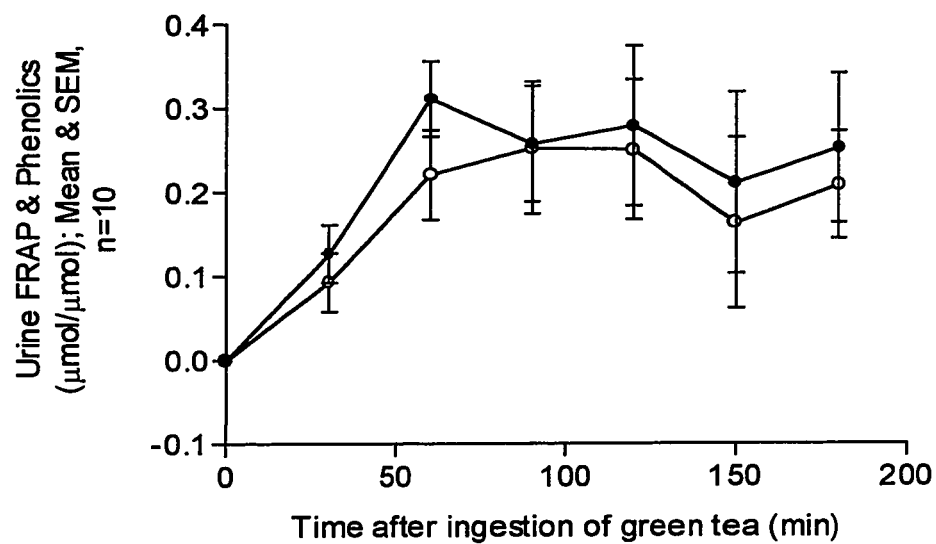


Figure 6.6 Urinary excretion (mean \pm SEM expressed as μmol per μmol creatinine) of antioxidant/reducing power, as FRAP, (filled symbols) and phenolics open symbols) after drinking green tea ($n=10$). Results show an increase in both FRAP and phenolics after ingestion of green tea.

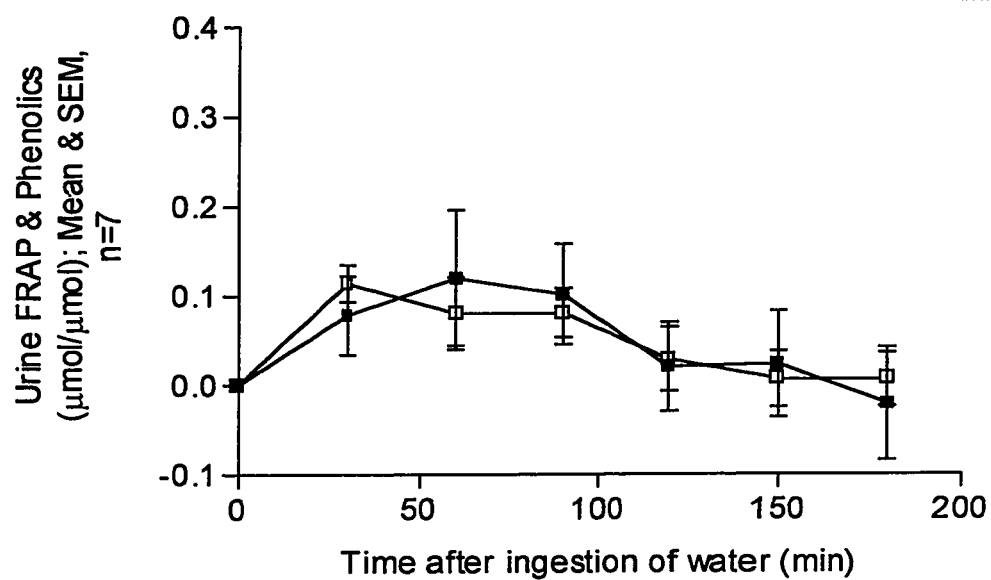


Figure 6.7 Urinary excretion (mean \pm SEM expressed as μmol per μmol creatinine) of antioxidant/reducing power, as FRAP, (filled symbols) and phenolics (open symbols) after drinking water ($n=7$). No increase in both FRAP and phenolics after ingestion of water.

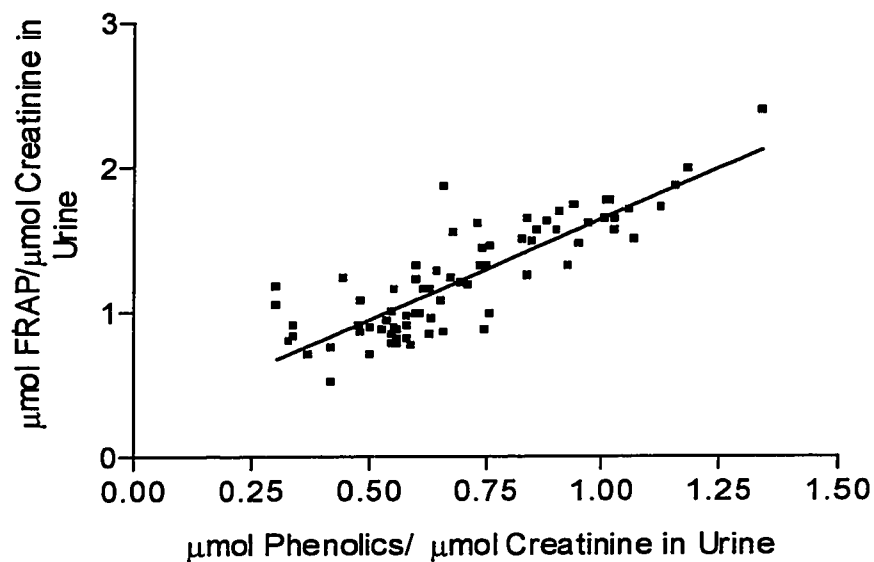


Figure 6.8 Relationship between urinary phenolics and antioxidant/reducing power, as FRAP, before and after drinking tea (squares). Results showed a significant, direct correlation between urinary phenolics and FRAP after ingestion of tea ($r=0.845$, 95% CI=0.766 to 0.900, $p<0.001$).

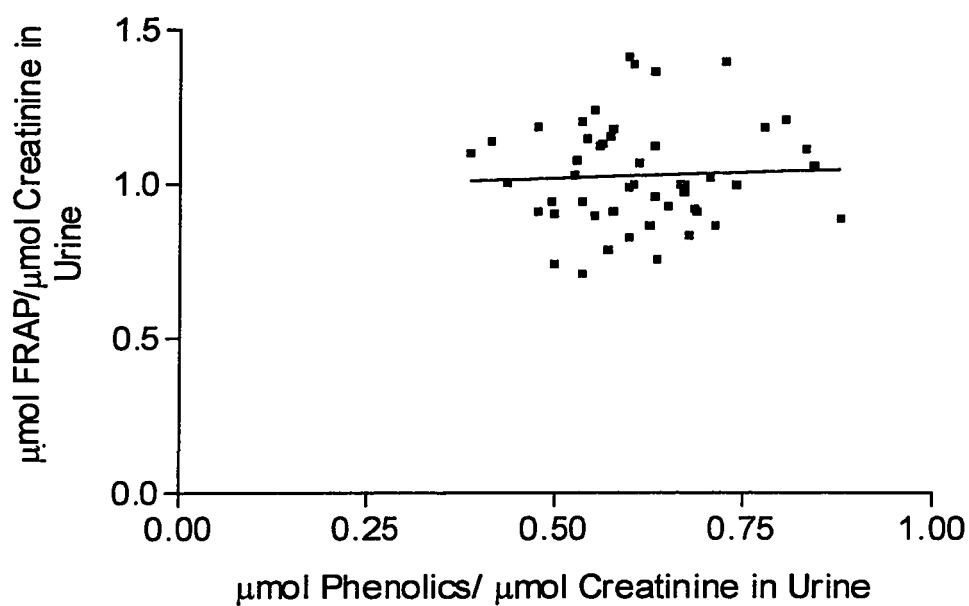


Figure 6.9 Relationship between urinary phenolics and antioxidant/reducing power, as FRAP, before and after drinking water. Results showed no significant correlation between urinary phenolics and FRAP after ingestion of water ($r=0.046$, 95% CI=-0.238 to 0.323, $p=0.752$). However only six data point available and no conclusive interpretation can be drawn.

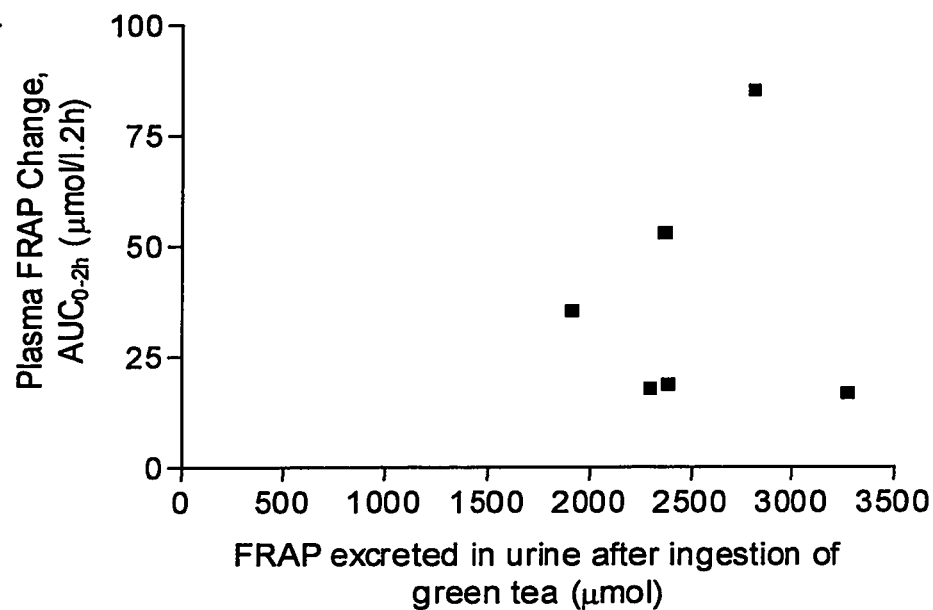


Figure 6.10 Correlation between antioxidant power (μmol FRAP) excreted in urine and plasma FRAP change (AUC_{0-2h}, μmol/l.2h) after ingestion of green tea (n=6). No association was found between these 2 parameters ($r=0.065$, 95% CI = -0.788 to 0.833, $p=0.903$), but numbers were small (n=6) and results therefore inconclusive.

CHAPTER 7 Antioxidant and Pro-oxidant Effects of the Chinese Herb Lingzhi, Antioxidant Vitamins, Wine and Tea Polyphenols Using the Comet Assay

Introduction

Both tea and red wine contain large amounts of polyphenolic antioxidants, and these can inhibit the peroxidation of low-density lipoprotein (Miura *et al.*, 1994; Abu-Amsha *et al.*, 1996; Ishikawa *et al.*, 1997; Luo *et al.*, 1997). Tea has a wide range of pharmacological properties (Hamilton-Miller, 1995; Dreosti *et al.*, 1997; Yang & Koo, 1997). Results of this study (Benzie & Szeto, 1999; Benzie *et al.*, 1999; for details see Chapters 5 and 6) have demonstrated that different types of tea have different antioxidant power and that at least some of the antioxidant power in green tea can be absorbed rapidly after ingestion. Wine is popular beverage with several thousands years of history (Soleas *et al.*, 1997) and moderate wine and alcohol consumption has been shown to be cardioprotective (Rimm *et al.*, 1991; Goldberg, 1995). Lingzhi (*Ganoderma lucidum*) is a traditional Chinese medicine which is reported to have hypoglycemic activity (Hikino *et al.*, 1989), anti-atherosclerotic properties (Li *et al.*, 1989) and radical scavenging effects (Wang *et al.*, 1985; Lin *et al.*, 1995). Preliminary study of Lingzhi showed a high total antioxidant activity; 12760 μmol of FRAP was found in 1 g of Lingzhi powder. Ascorbic acid and α -tocopherol are well known antioxidant vitamins. Ascorbic acid can react with and scavenge many kinds of free radicals such as singlet oxygen, superoxide, hydroxyl radicals, and can react directly with aqueous, chain-carrying peroxy radical (Bendich *et al.*, 1986). It is

thought that vitamin C can also regenerate the reduced forms of α -tocopherol and uric acid (Maples & Mason, 1988; Padh, 1991). Vitamin E is believed to help the maintenance of membrane integrity by stopping chain reactions among polyunsaturated fatty acids and reactive oxidising species (Combs, 1992). Benefits of these dietary and medicinal agents may be related to the contribution of their antioxidant activities. Uric acid is generally regarded as a waste product but it is also an effective *in vitro* antioxidant. It has been suggested that uric acid replaced ascorbic acid in primate evolution (Ames *et al.*, 1981; Cutler, 1991). Uric acid may, however, have pro-oxidant effects (Benzie & Strain, 1996a).

Antioxidants may help maintain health because cellular systems are challenged by reactive species and antioxidants minimise the damage caused by oxidative stress (Morrissey & O'Brien, 1998; Strain & Benzie, 1998b). Various antioxidants produced *in vivo* and/or obtained in the diet. Dietary antioxidants include ascorbic acid, α -tocopherol, carotenoids and flavonoids. Epidemiological findings have shown that intake of certain antioxidant-containing foods can lower the risk of oxidative-stress related disease, such as cancer and cardiovascular disease (Gao *et al.*, 1994; Yu *et al.*, 1995; Kushi *et al.*, 1996; Steinmetz, 1996; Gey, 1998). Both DNA oxidative damage and peroxidation are related to mutagenesis, increased risk of cancer (Emerit, 1994; Halliwell, 1996), and to atherogenesis and cardiovascular disease (Maxwell and Lip, 1997). However, while possessing antioxidant activities, pro-oxidant effect of some of flavonoids and ascorbic acid has been reported (Laughton *et al.*, 1989; Sakagami *et al.*, 1997). Further study into the DNA protective or damaging effect of these antioxidants on living cells is required.

One method of assessing DNA damage is the Comet Assay which was developed in 1988 by Singh and co-workers for detection of both single and double strand DNA breaks. The comet assay enables the detection of DNA strand breaks in individual cells (Singh *et al*, 1988). This test has also be used to study the *in vitro* and *in vivo* genotoxic or genoprotective effects of certain agents such as flavonoids (Anderson *et al*, 1995; Collins *et al* ,1997; Duthie *et al*, 1997; Panayiotidis & Collins, 1997).

Aims

The aim of this part of the study was to assess *in vitro* DNA protective or damaging effects of individual tea and wine polyphenols, infusions of tea (green and black) and Lingzhi powder infusion on human lymphocytes. The effect of ascorbic acid, α -tocopherol and uric acid at different concentrations was also investigated and compared.

Materials And Methods

Chemicals and reagents

Purest or molecular biology grade of the following chemicals were purchased:

For preparation of lymphocytes: RPMI 1640 and fetal bovine serum were from GiboBRL, Paisley, UK; Histopaque 1077, was from Sigma, St. Louis MO, USA.

For the comet assay: type VII low gelling point agarose, standard agarose, phosphate buffered saline tablets (PBS), sodium chloride, disodium

ethylenediaminetetraacetic acid dihydrate, Tris[hydroxymethyl]aminomethane, hydrogen peroxide solution, ethidium bromide, Triton X-100, uric acid, quercetin, caffeic acid, catechin, epicatechin, catechin gallate, epigallocatechin gallate, epigallocatechin, epicatechin gallate, resveratrol were from Sigma, St. Louis MO, USA; sodium hydroxide was from Riedel-de Haen, Germany; L-(+)-ascorbic acid, DL- α -tocopherol and hydrochloric acid were from Merck, Darmstadt, Germany; Unimate 5 uric acid test kit, Calibrator (human), Control serum P (human) were from Roche Diagnostic Co., Basel, Switzerland; green ('China green tea', Shanghai Tea Import and Export Corporation) and black ('China black tea', China National Native Product) tea leaves were purchased from a local shop; Lingzhi powder was a kind gift from Prof. Brian Tomlinson (Chinese University of Hong Kong, Hong Kong).

Preparation of test agents

Uric acid, ascorbic acid, α -tocopherol, Lingzhi, green and black tea were dissolved in PBS (0.01 mol/l, pH 7.4) and stored at 4°C in the dark for no more than 2 days before use (for uric acid and ascorbic acid, not more than 1 hour). Sonification was employed to assist dissolving if necessary. Uric acid and ascorbic acid solutions were freshly prepared each time. Five percent green and black tea infusion in PBS (w/v) were prepared as described in Chapter 5 except for diluent, and further diluted with PBS before testing. 0.02 % (w/v) of Lingzhi suspension was prepared by adding Lingzhi powder to 0.005 M NaOH and further diluted with PBS (0.01 M, pH 7.4). The mixture was then roller-mixed for 10 min, followed by centrifugation at 3700 rpm for 10 min (for further details of Lingzhi preparation, see Appendix 5). Concentrations of several test agents were checked

before and after 37°C incubation with lymphocytes. Ascorbic acid concentration was checked by FRASC assay (Benzie & Strain 1996b; Benzie & Strain 1999), α -tocopherol concentration was checked by measuring molar absorptivity at 292 nm and uric acid was checked by enzymatic method (Roche Diagnostic). For the purified polyphenols tested, one mmol/l stock solutions were prepared by dissolving solid polyphenol in PBS. This was stored at 4°C in the dark for up to 2 weeks until use. Concentrations of these polyphenols were not checked and nominal concentrations were assumed.

Preparation, harvesting and treatment of lymphocytes

The lymphocytes were harvested following the procedure of Duthie *et al.* with slight modification (Duthie *et al.*, 1997). Human lymphocytes from a healthy male subject (age 30) were isolated from fresh or 1 day old heparinised venous blood sample. Cells for the same subject, collected at various occasions, were used for all experiments. About 40 μ l of blood was added to 1 ml pre-chilled RPMI medium containing 10% fetal calf serum in a 1.5 ml microcentrifuge tube. Blood and medium were mixed gently and left on ice for 30 min before underlaying with 100 μ l Histopaque 1077. Tubes were spun at 2000 rpm for 5 min at 4°C. Lymphocytes were retrieved in 100 μ l from just above the boundary between the RPMI and Histopaque layers, using an autopipette. Retrieved lymphocytes were added to 1 ml chilled PBS in microcentrifuge tube. Centrifugation was repeated and as much supernatant as possible was removed using autopipette. One ml of PBS (with or without testing agents at various concentrations) was added to the tube and mixed with the cells. This was followed by incubation at 37°C for 30 min. The tubes were then centrifuged and cells

washed once with 1 ml PBS. For each sample, 0, 30, 45 and 60 $\mu\text{mol/l}$ H_2O_2 in PBS was used to induce oxidative stress immediately afterwards. One ml of each H_2O_2 solution was added to the lymphocytes in a tube. The tube was kept on ice for 5 min then spun. Cells were washed with 1 ml PBS and spun again. As much supernatant as possible was removed (for summary of procedure, see Figure 7.1) and the comet assay was performed on the treated, untreated, stressed and unstressed cells in parallel.

Single-cell Gel Electrophoresis (Alkaline Comet Assay)

The comet assay was performed following the procedure of Duthie *et al.* with slight modification (Duthie *et al.*, 1997) (for reagent preparation, see Appendix 8). Eighty-five μl of pre-warmed (40°C) 1 % low gelling point agarose made up in PBS was mixed with the washed cells (treated, untreated, stressed and unstressed) in microcentrifuge tube. The mixture was then immediately applied to the microscope slide which had been precoated previously with 85 μl of 1 % standard agarose in PBS. The slides were then placed at 4°C until gel layer solidified. Lysis solution (at 4°C) of 2.5 mol/l NaCl, 0.1 mol/l EDTA, 10 mmol/l Tris was adjusted to pH 10 by adding concentrated or solid NaOH. Five hundred μl of Triton X-100 was added to 50 ml of this solution just before use. Lysis of cells were performed by submerging the layered slides in a staining jar containing lysis solution in the absence of light for 1 hour at 4°C . Subsequently, slides were transferred to an electrophoresis tank (Sub-Cell GT, Bio-Rad) containing 0.30 mol/l NaOH and 1 mmol/l EDTA electrophoresis solution; slides were submerged in this fluid. DNA unwinding and expressing of alkali-labile sites was allowed for to proceed 40 min. Electrophoresis was then performed for 30 min at 25 V

constant voltage. The current was adjusted to 0.30 A by adjusting the electrophoresis solution level. After electrophoresis, slides were removed and neutralized by immersion in 3 changes (3 x 5 min) of 0.4 mol/l Tris at pH 7.5. Slides were then stained with 20 µg/ml ethidium bromide just before image analysis (for summary of procedure, see Figure 7.2).

Fifty cells were scored per treatment in each of 3 independent experiments (i.e. 150 cells for each single dose of treatment) using a fluorescence microscope (Optiphot-2, Nikon). Analysis of comets was performed by measuring the tail moment which is an index of DNA damage. Tail moment is the product of tail length and tail DNA content which measures the intensity of light of the comet tail and the proportion of DNA migrating into the tail. Computer image analysis system was used for tail moment measurement (Komet 3.0, Kinetic Imaging, Liverpool, UK).

Results

Results of testing agents with protective effect are shown in Figures 7.3-7.6. Decreased DNA damage of quercetin pre-treated lymphocytes after H₂O₂ stress was observed. 12.5 µmol/l quercetin solution was sufficient to show the protective effect. Caffeic acid also demonstrated the protective effect to DNA damage mediated by H₂O₂. But no clear dose-response was seen in caffeic acid protection. For α-tocopherol, a trend of decreased DNA damage was observed, but the effect was not so obvious and the protective effect is not convincing. Ascorbic acid at 50, 100 and 200 µmol/l showed a trend of decreased DNA damage caused by H₂O₂, however the effect was not strong enough to reach a

statistically significant level. All three of these testing agents showed no damaging effect on lymphocytes.

Figures 7.6 to 7.15 show the results of testing agents which resulted in a DNA damaging effect. Ascorbic acid and uric acid (Figures 7.7 & 7.8) started to damage DNA at 200 and 400 $\mu\text{mol/l}$ respectively and thereafter the response was positively related to dose applied. Resveratrol (Figure 7.9) also demonstrated a dose related DNA damage to cells while epigallocatechin, epigallocatechin gallate, epicatechin gallate (Figures 7.10 to 7.12) showed no dose-related response, i.e. a similar level of damage was seen irrespective of dose. Green tea had a higher DNA damage effect than black tea (Figures 7.13 & 7.14) but only black tea showed a strong positive dose-response. Lingzhi (Figure 7.15) was also found to damage DNA in cells but there was no obvious dose response at higher concentrations.

The flavanols (+) catechin, (-) catechin, catechin gallate and epicatechin (Figures 7.16 to 7.19) did not damage DNA, however, no DNA protection was seen in the pre-treated cells after 60 $\mu\text{mol/l}$ H_2O_2 challenge. The 30 and 45 $\mu\text{mol/l}$ H_2O_2 stress was not performed on these four polyphenols.

Discussion

Ascorbic acid and α -tocopherol are dietary antioxidants which are suggested to lower risk of certain diseases such as cancer and CHD (Odin, 1997; Benzie, 1998; Gey, 1998; Morrissey & O'Brien, 1998). The protection, by antioxidants, of DNA against oxidative stress may decrease the rate of mutation

and hence prevent cancer. A protective effect of α -tocopherol against DNA damage mediated by H_2O_2 was demonstrated in this part of study. This agreed with a previously reported study which used a lymphoblastoid cell line (Sweetman *et al.*, 1997). However in this current study no protective effect of ascorbic acid was seen even at lowest oxidative stress level ($30 \mu\text{mol/l } H_2O_2$) used, and the ability of ascorbic acid to lower DNA damage did not reach statistical significance. In addition, ascorbic acid at higher concentration ($> 200 \mu\text{mol/l}$) showed a DNA damaging effect. This is also in agreement with previously reported results and the ascorbyl radical has been suggested as the cause of the cytotoxic effect (Sakagami *et al.*, 1997). Human fasting plasma ascorbate concentration ranges from 45 to 90 $\mu\text{mol/l}$ (Gutteridge & Halliwell, 1994; Benzie *et al.*, 1998), but can approach 200 $\mu\text{mol/l}$ after a large oral dose (Benzie & Strain, 1997a). Moreover, intracellular ascorbic acid concentration can reach milimolar levels. Further study on the *in vivo* effect with regard to DNA damage and its physiological consequences is needed.

Uric acid had been proposed as an antioxidant (Niki *et al.*, 1985; Scott, 1997) but a pro-oxidant role of uric acid has also been suggested (Benzie & Strain, 1996a). Uric acid may consume the reducing power of ascorbic acid and lead to the insufficient reducing power for recycling of α -tocopherol. In this study, the pro-oxidant effect of uric acid on DNA was demonstrated and a concentration dependant response was shown. Generation of the superoxide anion and H_2O_2 generated by polymorphonuclear leukocytes in the presence of high concentration of uric acid has been reported (Thomas, 1992). DNA damage effect by uric acid shown in this study may also be mediated by the similar reaction. Fenton reaction

may occur when Fe^{2+} in the nucleus react with H_2O_2 and the hydroxyl radical attacks DNA molecules directly. The human serum level of uric acid is 150 – 450 $\mu\text{mol/l}$ and DNA damage in comet assay started at 400 $\mu\text{mol/l}$, which is below the upper limit of normal range. It should be noted that uric acid in PBS *in vitro* may not have the same behaviour as *in vivo* but the role and action of uric acid as a pro-oxidant or antioxidant may depend on the situation.

Flavonoids are polyphenolic compounds which can be found in foods of plant origin. The antioxidant activity of flavonoids is due to the hydrogen donating property of the hydroxyl group and the antioxidant power is related to the number and positions of hydroxyl groups (Rice-Evans *et al.*, 1995; Cao *et al.*, 1997). Flavonol is the subclass of flavonoids and the most representative compound of this class is quercetin, which is a well studied antioxidant (Herrmann, 1976; Hollman & Katan, 1997) and is the major flavonol in the diet. Increased concentration and antioxidant capacity in blood after ingestion of quercetin rich foods or beverage has been demonstrated (Day *et al.*, 1997; Morand *et al.*, 1998; de Vries *et al.*, 1998). Quercetin is an effective inhibitor of LDL oxidation (de Whalley *et al.*, 1990) and is a scavenger of superoxide anion (Robak & Gryglewski, 1988). A recent study showed the metabolites of quercetin in plasma of rats also possess antioxidant properties (Morand *et al.*, 1998). Although pro-oxidant effect on DNA of quercetin has been reported (Laughton *et al.*, 1989), our results showed that quercetin pretreatment can decrease the damage of human lymphocyte DNA induced by hydrogen peroxide, which agrees with previous studies (Musonda & Chipman, 1998; Noroozi *et al.*, 1998)

Caffeic acid is a non-flavonoid phenolic compound found in vegetables and fruits (Hermann, 1989). Inhibition of Cu^{2+} -induced, hydrophilic azo initiator induced and myoglobin-catalysed LDL oxidation by caffeic acid has been shown (Laranjinha *et al.*, 1994; Vieira *et al.*, 1998). However caffeic acid was shown to be carcinogenic when incorporated into the diet at high concentration in an animal model (Lutz *et al.*, 1997). The lesions induced by caffeic acid were reversible, unlike those induced by genotoxic carcinogens (Ito & Hirose, 1989). Guanine oxidation of rat DNA was also observed in high caffeic acid diet (Zhizhina & Blyukhterova, 1997). Moreover, with the presence of transition metals such as Mn and Cu, DNA damage in cultured cells was induced by caffeic acid (Inoue *et al.*, 1992). This was probably due to the formation of hydrogen peroxide followed by activation by transition metals (Inoue *et al.*, 1992).

Red wine and tea are rich dietary sources of antioxidant power. These plant based compounds contain quercetin, catechin, gallic acid and resveratrol (Miller & Rice-Evans, 1995). *In vitro* radical trapping antioxidant properties of black tea and green tea, of extracts of tea, and of individual polyphenolic compounds found in tea, have been demonstrated (Cook & Samman, 1996; Zhao *et al.*, 1989; Scott *et al.*, 1993; Lin *et al.*, 1996; Paganga *et al.*, 1996; Rice-Evans *et al.*, 1996; Zhang and Shen, 1997). It is worth noting that, tea polyphenolics are known to bind metal ions (Miller *et al.*, 1996; Paganga *et al.*, 1996; Van Acker *et al.*, 1996). Binding of metal ions, such as iron, *in vivo* is an antioxidant action as it prevents metal ion catalysed generation of reactive species (Emerit, 1994; Halliwell, 1996). However, tea polyphenolics also have electron donating antioxidant properties, the relative activity of the different polyphenolic

compounds being related to the number and location of the hydroxyl groups and the presence of the galloyl moiety (Lin *et al.*, 1996; Miller *et al.*, 1996; Paganga *et al.*, 1996; Rice-Evans *et al.*, 1996). The resultant effect of pro-oxidant or antioxidant properties may depend on the amount of metal already bound to the polyphenol.

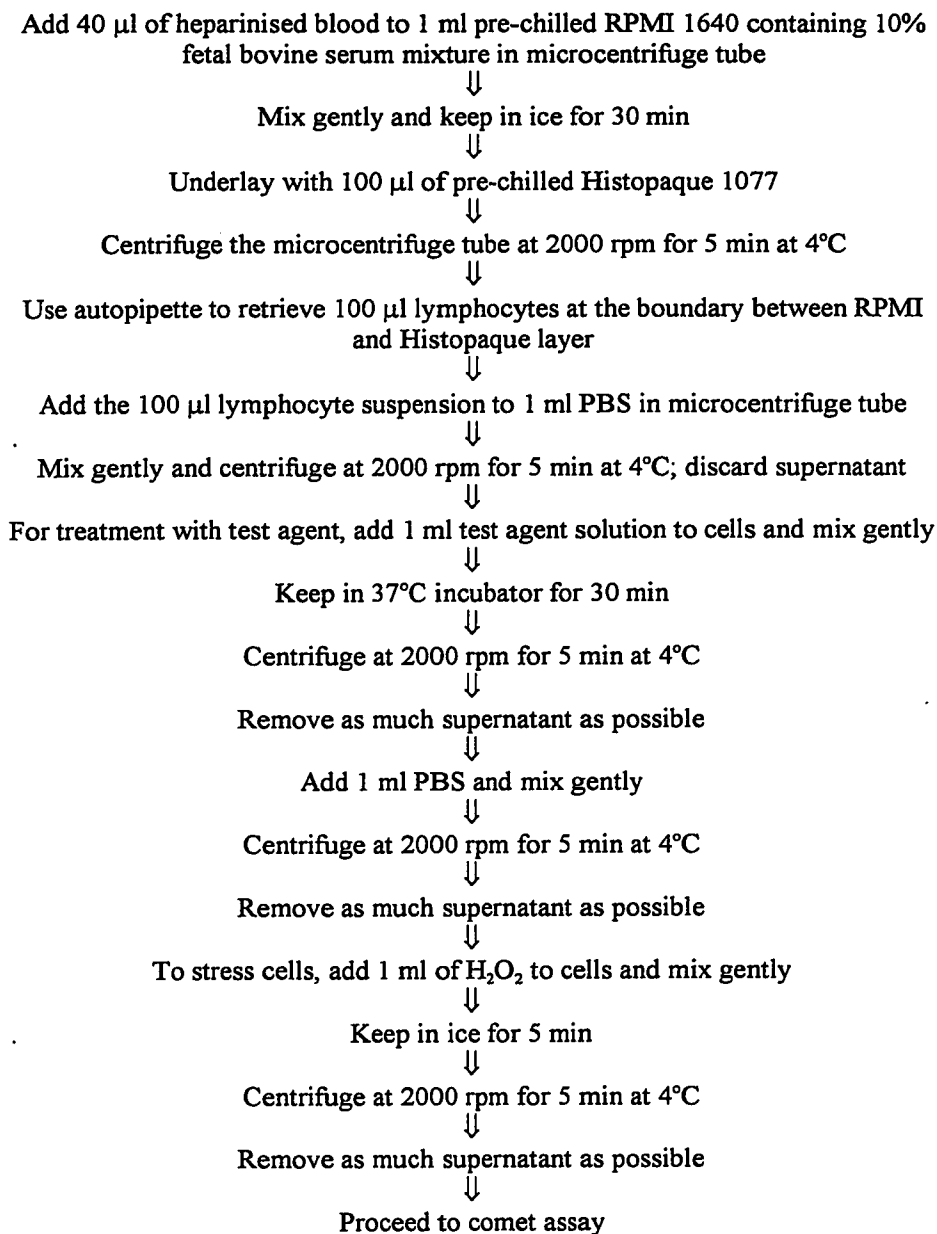
Results of this study indicate that the protective effect of antioxidant-rich diets may be mediated by a small number of antioxidants only, and that some antioxidants may have undesirable DNA damaging effects either directly, or perhaps owing to iron binding. The effect of DNA protection or damage to human lymphocytes and other cells after *in vivo* supplementation with various polyphenolics is worth further study, particular with those which showed protective effects (quercetin and caffeic acid). Those agents tested which showed a damaging effect may best to be further tested using cell culture techniques or animal studies.

In conclusion, this part of the study presents information comparing the *in vitro* DNA protective and damaging effect of antioxidant vitamins, uric acid, tea and wine polyphenols using the comet assay. Results indicate that quercetin, caffeic acid and α -tocopherol were protective against oxidant stress. Ascorbic acid at low concentration ($< 200 \mu\text{mol/l}$) showed a trend of protection but this did not reach statistical significant level. Catechin, epicatechin and catechin gallate showed no apparent DNA protective or damaging effect. Uric acid and ascorbic acid at high concentration ($> 200 \mu\text{mol/l}$), and epigallocatechin, epigallocatechin

gallate, resveratrol, green tea, black tea and Lingzhi at all concentrations tested caused increased DNA damage.

Data presented in this chapter have been accepted for presentation at:

The 17th International & 13th European Congress of Clinical Chemistry & Laboratory Medicine, 1st International Congress of Clinical Molecular Biology (CMB), 31st National Congress of the Italian Society of Clinical Biochemistry and Clinical Molecular Biology, 6-11 June 1999, Firenze, Italy. (with IFF Benzie, B Tomlinson)



- All cells following the same procedure including unstressed or untreated cells. PBS was used instead of H₂O₂ or test agent at the relevant stages.

Figure 7.1 Summary of protocol for harvesting and treatment of lymphocytes

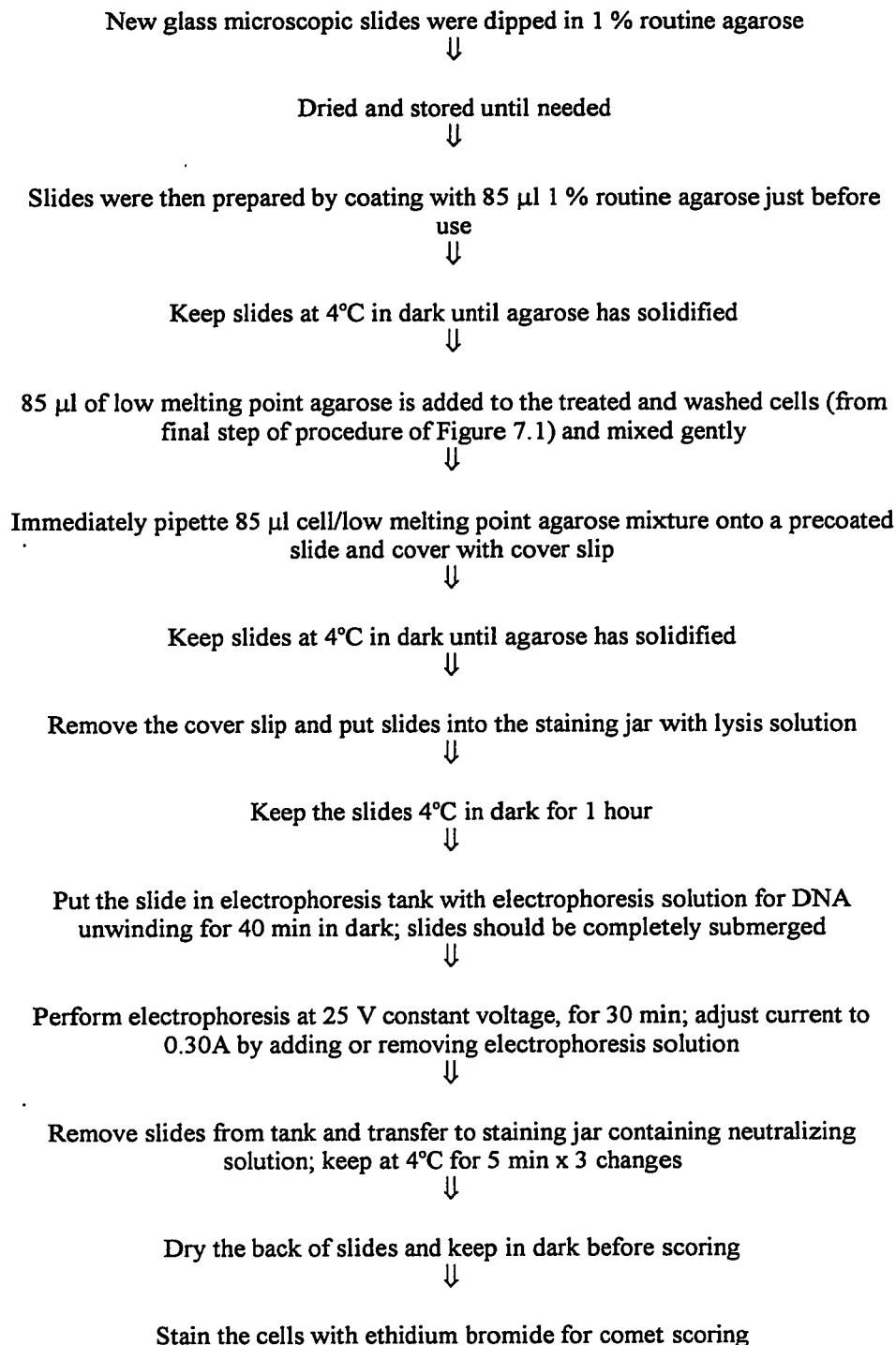


Figure 7.2 Summary of protocol for the comet assay procedure

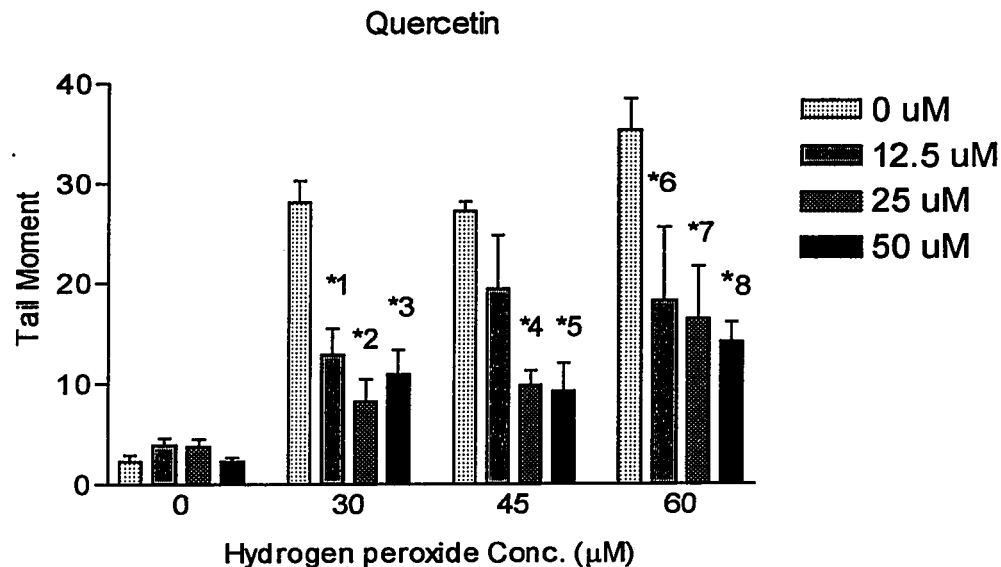


Figure 7.3 Effect of quercetin pre-treatment on H_2O_2 -mediated DNA damage on lymphocytes. Lower H_2O_2 -mediated DNA damage was shown by decreased tail moment indicate the protective effect of quercetin pre-treatment. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. No damaging effect was seen at increasing quercetin concentration in unstressed cells. Tail moment score increased to 30-40 with H_2O_2 -induced stress in cells not pre-incubated with quercetin. Damage was less in cells pre-treated with quercetin, although no clear dose-response was seen in this protective effect. Tail moments of treated cells were compared to that of the untreated cells under the same level of H_2O_2 stress.

- *1 $P=0.008$
- *2 $P<0.0001$
- *3 $P=0.0003$
- *4 $P<0.0001$
- *5 $P=0.0003$
- *6 $P=0.0409$
- *7 $P=0.0076$
- *8 $P<0.0001$

Caffeic acid

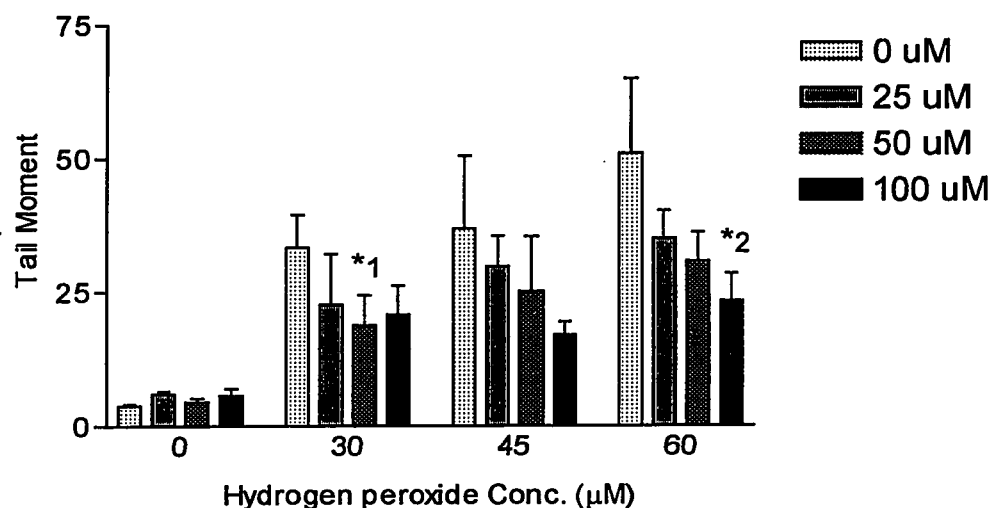


Figure 7.4 Effect of caffeic acid pre-treatment on H_2O_2 -mediated DNA damage on lymphocytes. Lower H_2O_2 -mediated DNA damage was shown by decreased tail moment indicate the protective effect of caffeic acid pre-treatment. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. No damaging effect was seen at increasing caffeic acid concentration in unstressed cells. Tail moment score increased to 30-50 with H_2O_2 -induced stress in cells not pre-incubated with quercetin. Damage was less in cells pre-treated with caffeic acid. No clear dose-response was seen in this protective effect, but is indicated by decreased tail moment at higher H_2O_2 concentrations. Tail moments of treated cells were compared to that of the untreated cells under the same level of H_2O_2 stress.

*1 $P=0.0238$

*2 $P=0.0488$

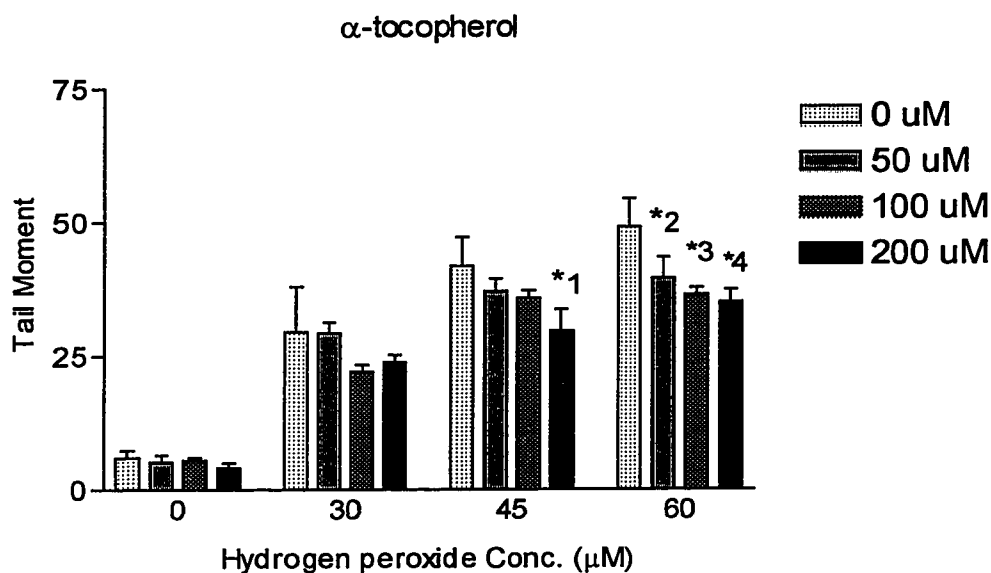


Figure 7.5 Effect of α -tocopherol pre-treatment on H_2O_2 -mediated DNA damage on lymphocytes. Lower H_2O_2 -mediated DNA damage was shown by decreased tail moment indicates the protective effect of α -tocopherol pre-treatment. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. No damaging effect was seen at increasing α -tocopherol concentration in unstressed cells. Tail moment score increased to 30-50 with H_2O_2 -induced stress in cells not pre-incubated with quercetin. Trend of decreased damage was seen in cells pre-treated with α -tocopherol, but the protective effect was not convincing. Tail moments of treated cells were compared to that of the untreated cells under the same level of H_2O_2 stress.

- *1 P=0.0447
- *2 P=0.0390
- *3 P=0.0050
- *4 P=0.0080

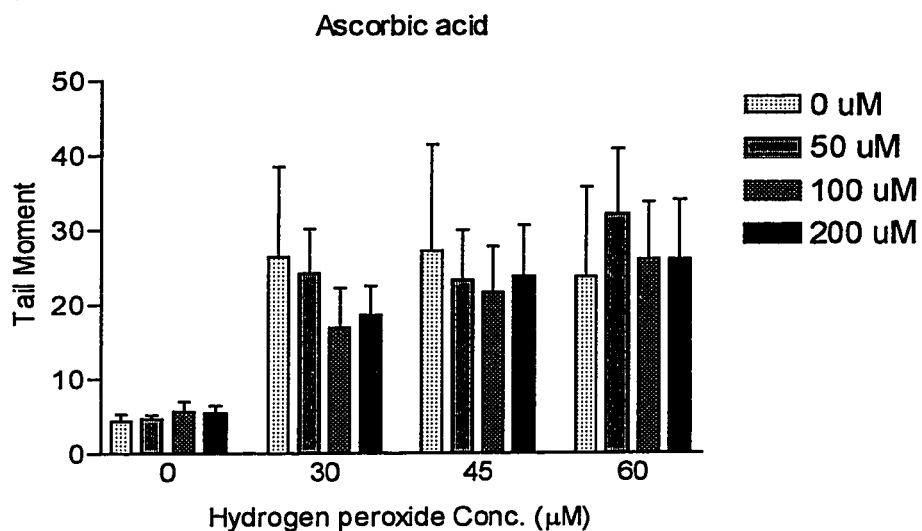


Figure 7.6 Effect of ascorbic acid pre-treatment on H_2O_2 -mediated DNA damage on lymphocytes. Trend of lower H_2O_2 -mediated DNA damage was shown by decreased tail moment but did not reach statistically significant level. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells under the same level of H_2O_2 stress.

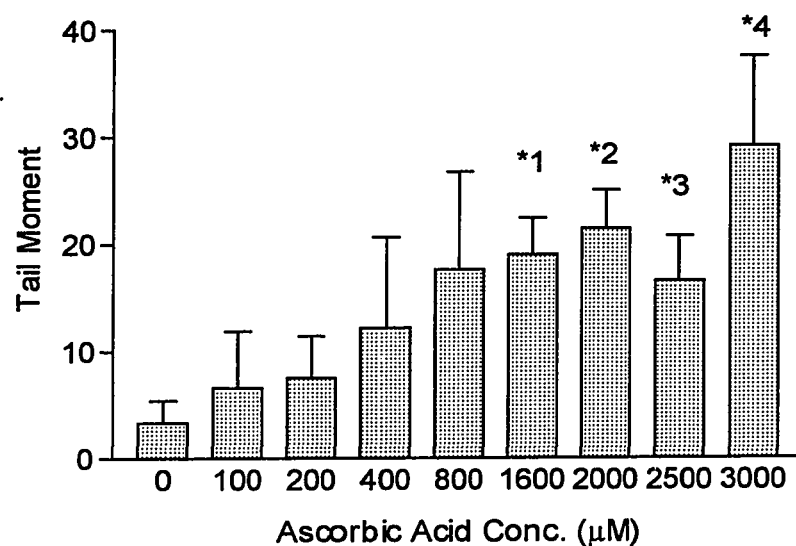


Figure 7.7 Effect of high dose ascorbic acid pre-treatment on lymphocytes. DNA damage was found to be positively related to dose. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

*1 P=0.0025

*2 P=0.0017

*3 P=0.0079

*4 P=0.0066

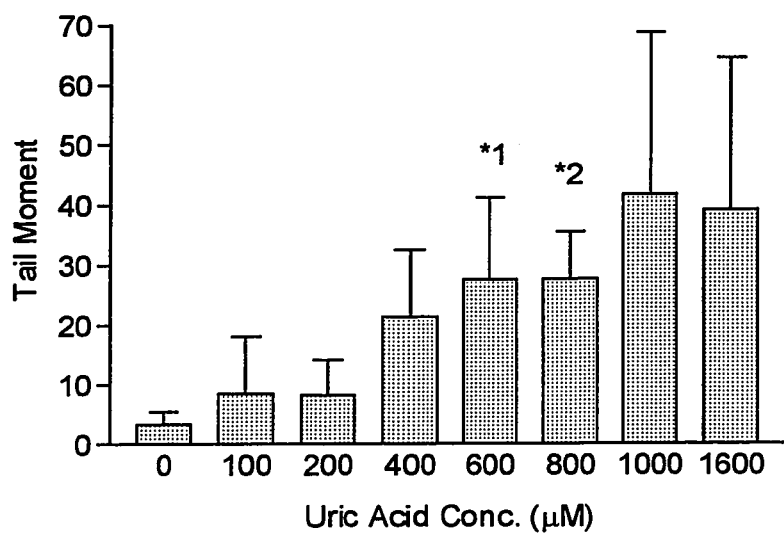


Figure 7.8 Effect of high dose uric acid pre-treatment on lymphocytes. DNA damage was found to be positively related to dose. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

*1 P=0.0381

*2 P=0.0066

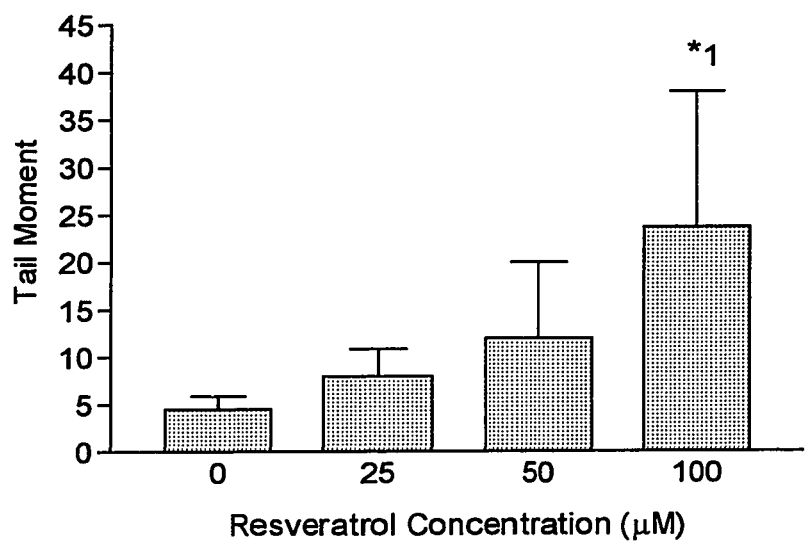


Figure 7.9 Effect of resveratrol pre-treatment on lymphocytes. DNA damage was found to be positively related to dose. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

*1 P=0.0367

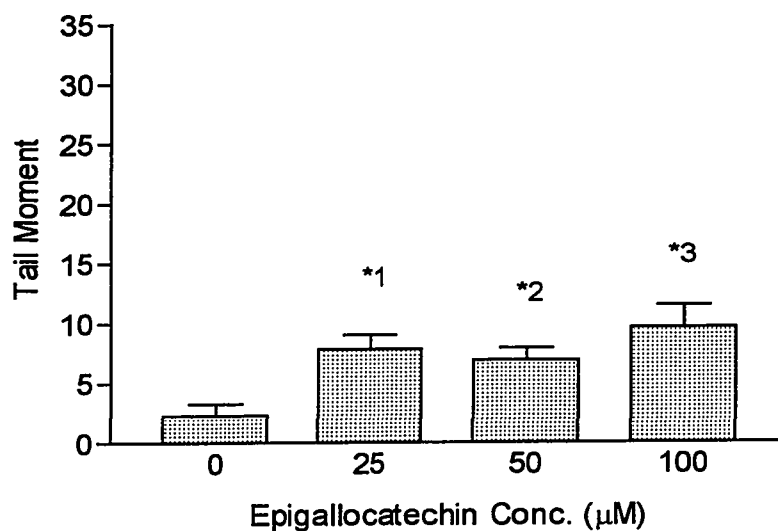


Figure 7.10 Effect of epigallocatechin pre-treatment on lymphocytes. DNA damage was found but no obvious dose-response was seen. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

*1 P=0.0034

*2 P=0.0045

*3 P=0.0036

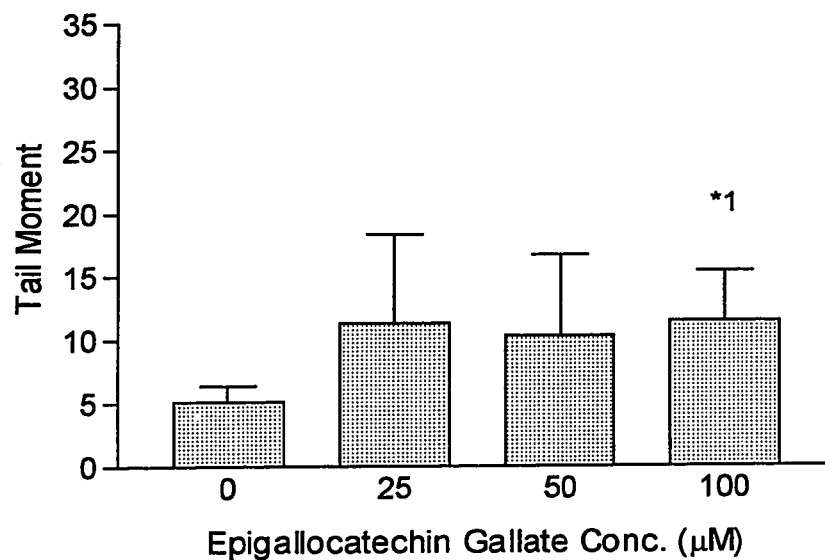


Figure 7.11 Effect of epigallocatechin gallate pre-treatment on lymphocytes. DNA damage was found but no obvious dose-response was seen. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

*1 P=0.0056

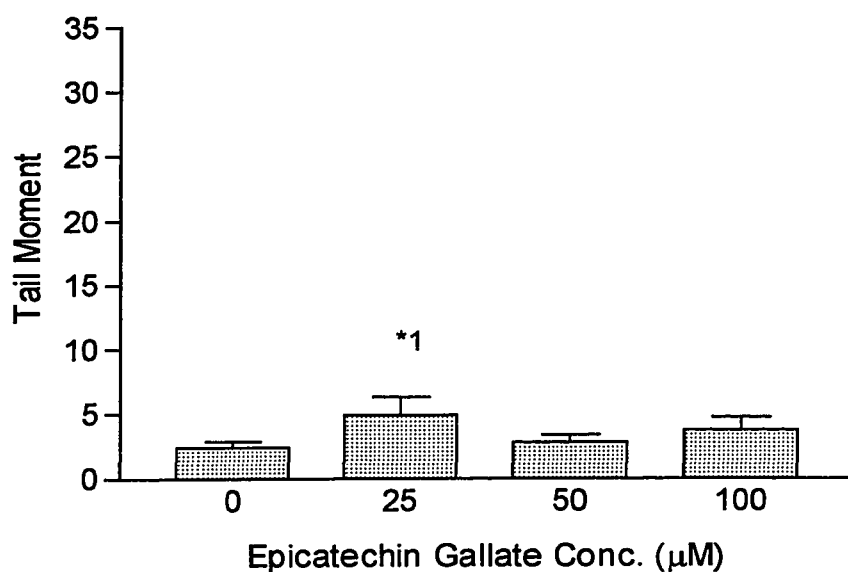


Figure 7.12 Effect of epicatechin gallate pre-treatment on lymphocytes. DNA damage was found but no obvious dose-response was seen. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

*1 P=0.0428

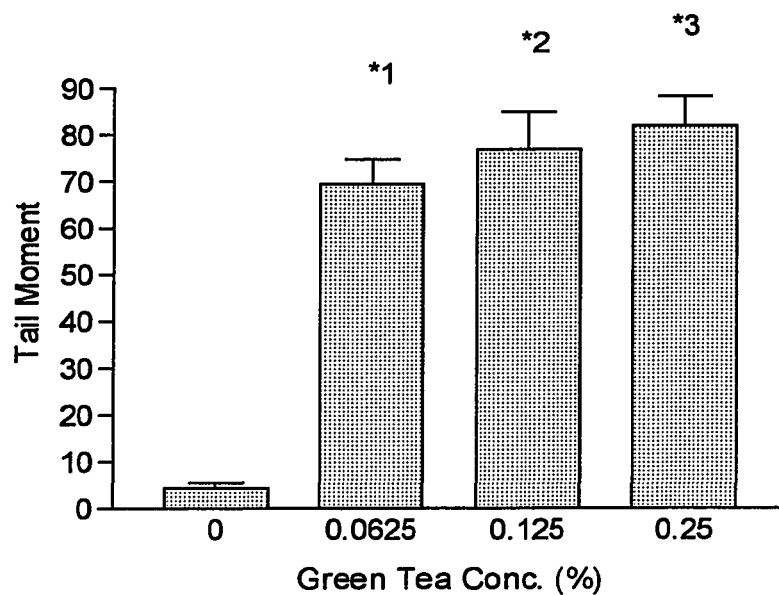


Figure 7.13 Effect of green tea pre-treatment on lymphocytes. DNA damage was found with a weak dose-response seen. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

*1 $P < 0.0001$
*2 $P < 0.0001$
*3 $P < 0.0001$

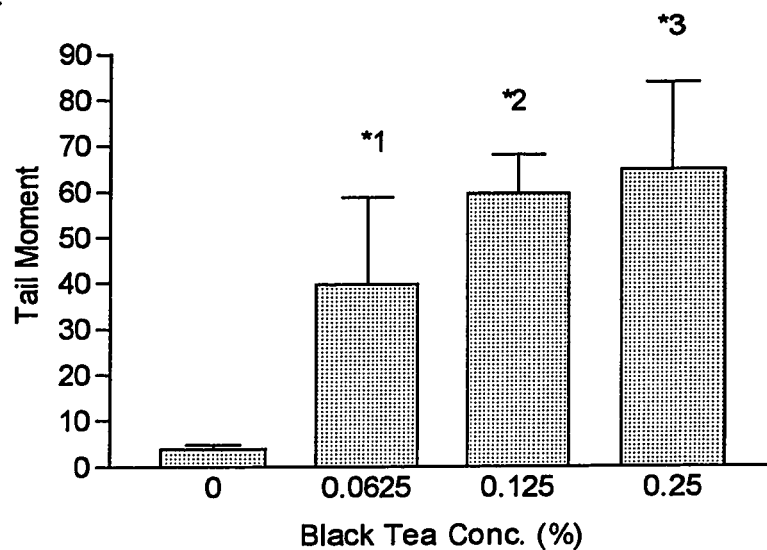


Figure 7.14 Effect of black tea pre-treatment on lymphocytes. DNA damage was found to be positively related to dose. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

*1 $P=0.0317$

*2 $P=0.0003$

*3 $P=0.0053$

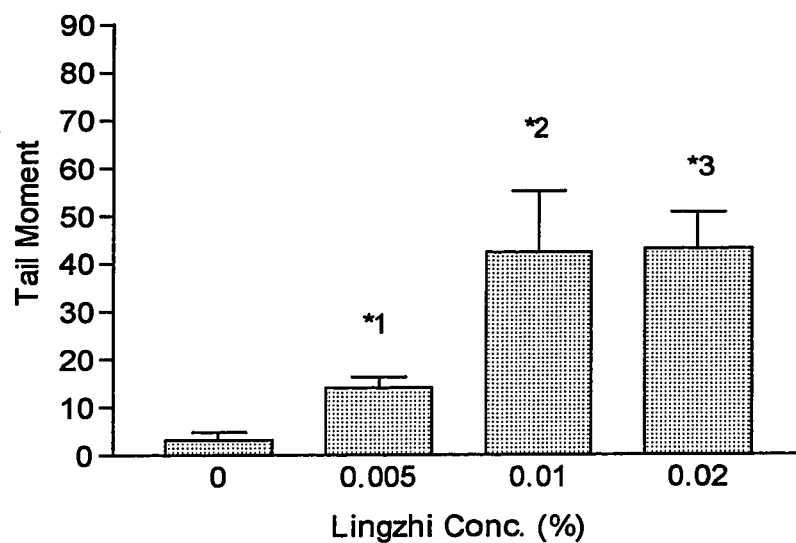


Figure 7.15 Effect of lingzhi pre-treatment on lymphocytes. DNA damage was found to be positively related to dose but reach a plateau at high concentrations. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

*1 P=0.0024
*2 P=0.0064
*3 P=0.0009

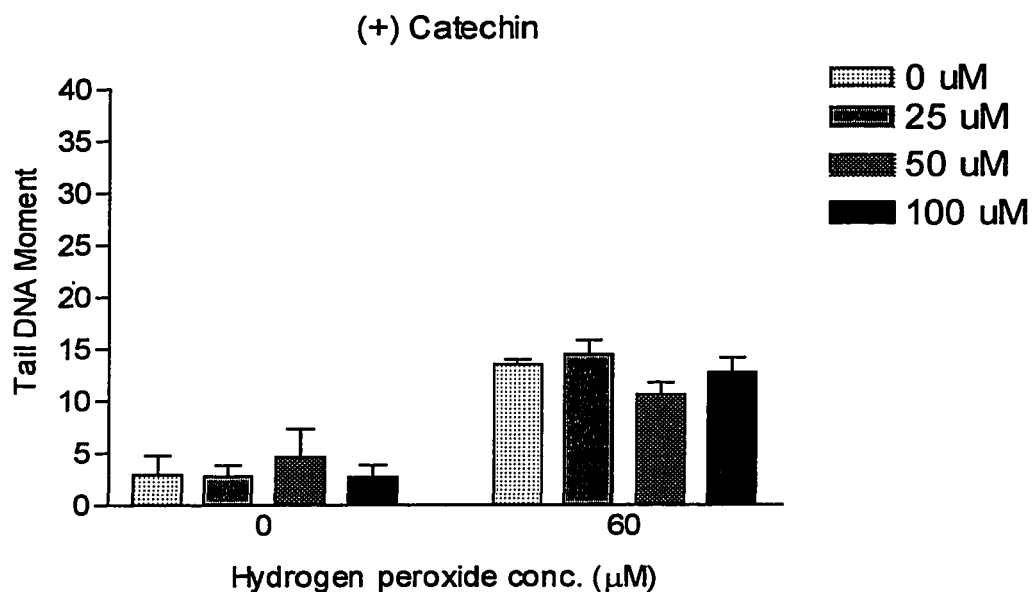


Figure 7.16 Effect of (+)-catechin pre-treatment on H_2O_2 mediated DNA damage on lymphocytes. No DNA protective or damage was found. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

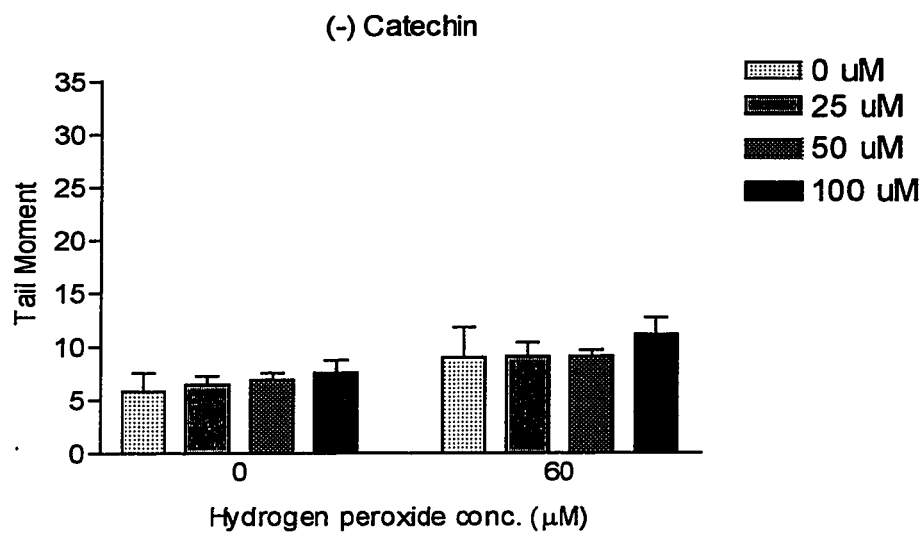


Figure 7.17 Effect of (-)-catechin pre-treatment on H_2O_2 mediated DNA damage on lymphocytes. No DNA protective or damage was found. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

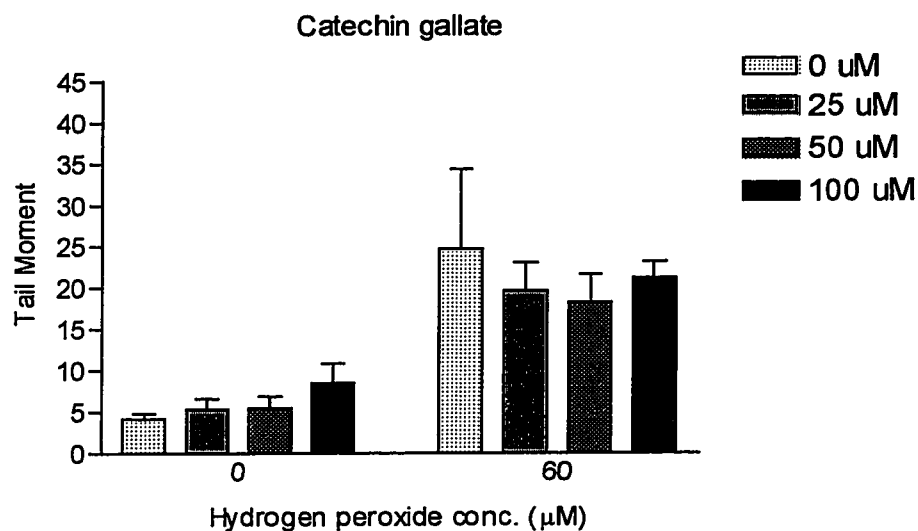


Figure 7.18 Effect of catechin gallate pre-treatment on H_2O_2 mediated DNA damage on lymphocytes. No DNA protective or damage was found. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

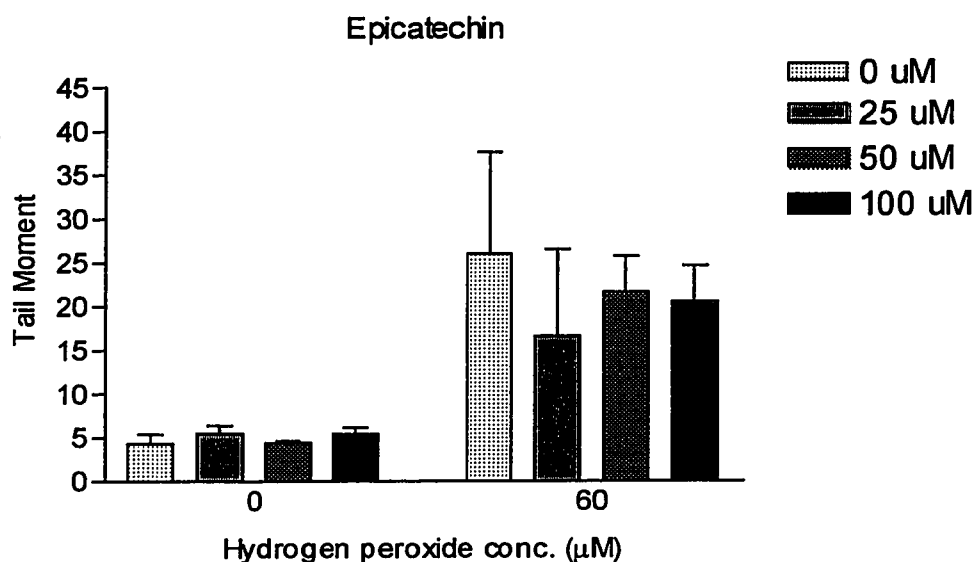


Figure 7.19 Effect of epicatechin pre-treatment on H_2O_2 mediated DNA damage on lymphocytes. No DNA protective or damage was found. Large variation in stressed cells however was seen. Each bar represents the mean of at least 3 individual experiments with 1SD error bars shown. Tail moments of treated cells were compared to that of the untreated cells.

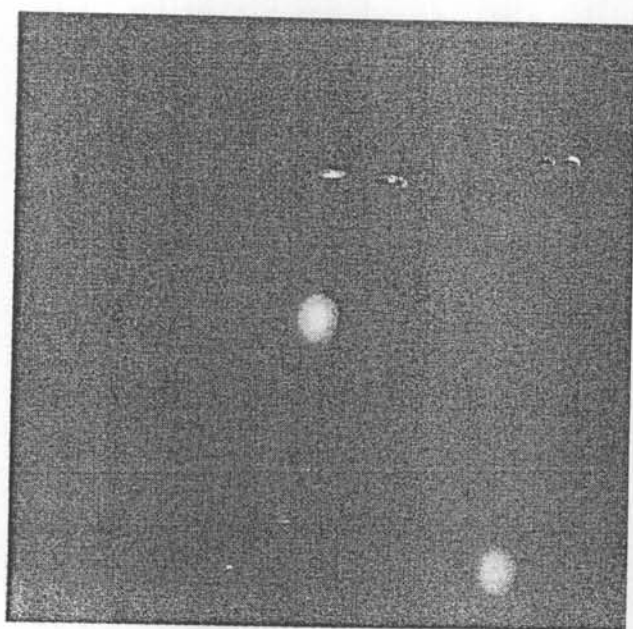


Figure 7.20 Typical undamaged lymphocyte nucleus after the comet assay

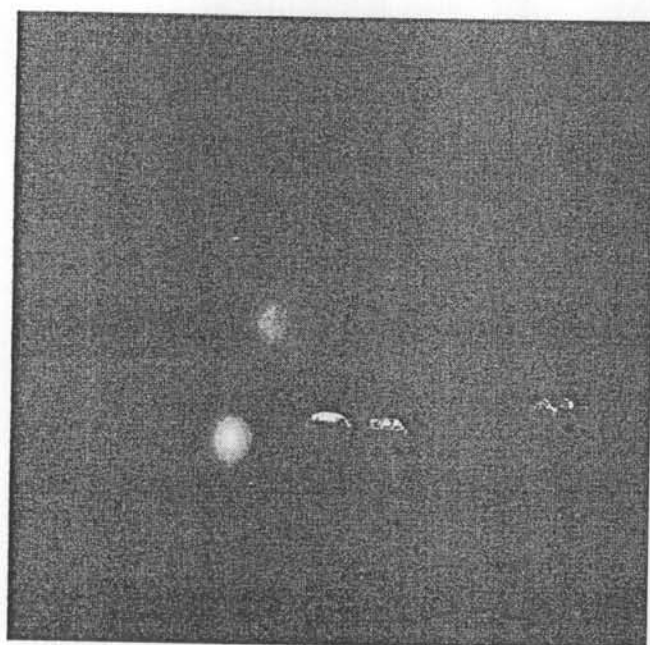


Figure 7.21 Typical damaged lymphocyte nucleus (comet) after the comet assay

CHAPTER 8 CONCLUSIONS, FINAL COMMENTS AND SUGGESTIONS FOR FURTHER WORK

In this study, the total antioxidant power of various drugs, fruits and vegetables and different types of tea was studied. Tea was found to contain a large amount of polyphenolic compounds and possess high level of total antioxidant power. A green tea therefore was selected for the human absorption study. In addition, individual tea and wine polyphenols and other antioxidants were selected for the investigation of *in vitro* DNA protective or damaging effects, assessed using the comet assay.

Initial screening of antioxidant power of oral hypoglycaemic and lipid lowering drugs was performed. Some drugs showed high antioxidant activities. Although the measured antioxidant power may not be directly derived from the active drug itself, ingestion of the drug with the intrinsic or added antioxidant power of packing materials / fillers may lead to additional beneficial therapeutic effect, and this deserve further study. Drugs under same category share the same primary pharmacological effect, such as statins used for lowering the plasma cholesterol level. Comparing the *in vivo* antioxidant power effect during treatment between different members of the same category showing antioxidant properties would be useful to investigate if there is an additional beneficial role of selected medicinal agents in terms of lowering oxidative stress. Results of this current study indicate that gliclazide and simvastatin, used in Type 2 (NIDDM) diabetes and hypercholesterolaemia, respectively, have *in vitro* antioxidant power. If this

characteristic is found to improve *in vivo* antioxidant defence then, if all other clinical considerations are equal, these may be the drugs of choice in their pharmacological category. The effectiveness of this additional beneficial effect remains to be confirmed and clinical trials investigating drug-related changes in antioxidant status are suggested.

The second part of the study was on fruits and vegetables. The compilation of a database of antioxidant power and ascorbic acid level of a range of fruits and vegetables, including some Chinese varieties, was commenced. In order to develop effective dietary strategies to improve antioxidant status and delay the development of chronic diseases, investigation of the antioxidant power of the foods making up the diet is the first step. Current food tables list the content of antioxidant vitamins, but information of non-vitamin antioxidants is not available and food tables require constant updating (Holland *et al.*, 1991). It is essential to know the total antioxidant capacities of individual foods before recommendation regarding the types and quantity of foods to be taken can be made. Fruits, vegetables, wines and teas are known to contain antioxidant-rich components, but systematic study of their antioxidant capacities has not yet emerged. The development of a database or a 'new' food table of total antioxidant power assists us to plan the intake of antioxidants in a more feasible and realistic manner.

Results of this current study have shown that different types of fruits and vegetables have widely different levels of antioxidant content, and that storage and processing effects can lead to significant decreases in their antioxidant power and ascorbic acid content. This has implications for planning antioxidant rich diets

in the event that these are confirmed to be beneficial to health, and for processing and storage recommendations and quality control in food manufacturing. For the development of the dietary antioxidant database, more can be done on the fruits and vegetables. Further study can be performed to assess the antioxidant content and profile of fresh, stored, processed and cooked food, as well as of tropical, oriental, and genetically modified varieties not previously studied. Those results will facilitate the planning of diets with optimum amount of antioxidants, and methods of food preparation and storage which minimise antioxidant loss could be established and used. Food listed in the published food tables may also be tested for total antioxidant power, since this novel parameter will be useful if a definite relationship between disease, health and total antioxidant power emerge. The end result could be a more realistic and achievable dietary target of antioxidant intake, with the realisation of potential health benefits.

The last part of the *in vitro* total antioxidant determination was on teas. This part of study has verified that the antioxidant power of green tea is much higher than black tea, while Oolong tea appeared similar to black tea. The herbal tea is not the traditional tea plant (*Camellia sinensis*) and contains lowest antioxidant power. Nevertheless, as this is of plant origin, herbal tea does contain significant amounts of antioxidant power. Lingzhi (*Ganoderma Lucidum*), a Chinese herbal remedy, also contains a substantial amount of antioxidant power. Bioavailability and benefits, however, remain to be established.

While knowing the content of antioxidant power in the foods is useful, information regarding the absorption of these antioxidants is also critical. The

value of the food in terms of antioxidant content will be largely irrelevant if the antioxidants present cannot be absorbed by the human body. In this study, a beverage (i.e. green tea) which contains very high *in vitro* antioxidant power was selected for a human absorption ('bioavailability') test. Results of this study of absorption of green tea antioxidants showed that at least some polyphenolic antioxidant compounds in green tea can be absorbed and enter the systemic circulation rapidly after ingestion, and that the absorption of these polyphenolics causes a small but significant increase in plasma antioxidant power. Although the absorption of tea antioxidant was relatively low, the contribution from tea cannot be neglected since tea is consumed regularly and in large quantities worldwide and regular consumption of green tea may result a small but worthwhile improvement in antioxidant status (Balentine, 1992). The low level of absorption of green tea antioxidants may be owing to the selective absorption of certain groups of antioxidants or to inactivation of the absorbed antioxidants *in vivo*. The bioavailability of antioxidants in black or oolong teas and the saturation limit of tea antioxidants absorption are still not known. Individual polyphenols may elicit different response, and some may be more easily absorbed than others. These aspects require further study.

The key constituents in tea which contribute to the spike of antioxidant power in blood remain to be investigated. Previous tea catechins and quercetin absorption studies generally demonstrated the time of maximum level of quercetin or individual catechins in blood was longer than one hour post-ingestion (Summary of studies, see Tables 8.1-8.2). While those studies that involved the measurement of total antioxidant power, including this current study, showed that

the maximum antioxidant power was found to be at less than one hour. A similar time frame was reported in a study of response to drinking red wine (Serafini *et al.*, 1996, Duthie *et al.*, 1998; Serafini *et al.*, 1998; Benzie *et al.*, 1999; Summary of studies, see Tables 8.1 & 8.3). Hence the contribution of antioxidant activity by tea may not be mediated by catechins. The possible suggested candidates are rutin, myricetin, gallic acid and caffeic acid since they are all found in black tea, green tea and red wine. (Balentine, 1992; Teissedre *et al.*, 1996).

This was the first study to use the FRAP assay as a 'bioavailability' tool, and other studies investigating absorption, distribution and excretion of dietary antioxidants can be modelled on this in future studies. Whether regular consumption of green tea leads to a significant response in terms of decreased oxidative damage to DNA and lipid deserves further study, especially since some of the tea polyphenols were found to exert pro-oxidant effects in the *in vitro* study of DNA damage/protection using the comet assay.

It is also worth identifying the absorbed and active antioxidant components in tea which may help to explain the inconsistency of the beneficial effect of tea as reported in the literature (Goldbohm *et al.*, 1996; Kohlmeier *et al.*, 1997). In this study, the *in vitro* effect of individual tea antioxidants and other antioxidants on DNA was also investigated. Surprisingly, many of the polyphenols found in tea showed a pro-oxidant effect instead of a DNA protective effect. Results indicate that quercetin, caffeic acid and α -tocopherol are protective against oxidant stress. Ascorbic acid at low concentration showed a trend of protection but this did not reach a statistically significant level. Uric acid and ascorbic acid at high

concentration, and epigallocatechin, epigallocatechin gallate, resveratrol, green tea, black tea and Lingzhi at all concentrations tested caused increased DNA damage. Results imply that the apparently protective effect of antioxidant-rich diets may be mediated by a small number of antioxidants only. Some antioxidants may have an undesirable DNA damaging effect. This may also be the reason that the effect of drinking tea is not conclusive from epidemiological study since different teas have different polyphenol profiles (Kohlmeier *et al.*, 1997; Wiseman *et al.*, 1997).

The protective or damaging effect on DNA using human lymphocytes and other cells after *in vivo* supplementation with various polyphenolics is worth further investigation. *In vitro* protective effects may not necessarily be seen *in vivo*. Similarly, those agents tested which showed a damaging effect *in vitro* may not be genotoxic *in vivo*. However, it may be best to study those which showed DNA damaging effects using cell culture techniques or animal studies in the first instance. However, undesirable damaging effect on DNA does not mean the same effect will result on other types of biomolecule. Protection and damage may be site specific. Further, the absolute concentration of the antioxidant may be critical to the 'pro-oxidant' effect of the 'antioxidant'. Co-operation between antioxidants may be crucial, and increasing the level of a single antioxidant may disturb the integrated system and may not be a successful strategy. This could help account for the strong beneficial effect of high intake of fruits and vegetable in epidemiological studies while inconsistent results have been obtained from vitamin supplementation studies (Benzie, 1998; Gey, 1998). ATBC and CARET studies are examples in which undesired effect of vitamin supplementation were observed (Albanes *et al.*, 1995; Albanes *et al.*, 1996; Omenn *et al.*, 1996).

The antioxidant capacity of a compound is a composite of different actions such as reducing power, radical scavenging and metal chelating effects. Many diverse antioxidants exist *in vivo*, hence no single test can give the full picture of antioxidant capacity. In this study, the FRAP assay was used to measure *in vitro* antioxidant power of selected drugs, foods and biological samples. Although this assay cannot show all the facets of antioxidant capacity of a compound, it is an objective and highly reproducible tool for the initial screening of 'antioxidant' (electron donating, reducing) capacity and for monitoring the changes within an individual after ingestion of a putative antioxidant compound, i.e. as a bioavailability tool. For the detailed study of particular substances, other assays such as the indirect radical trapping methods of measurement of antioxidant capacity could be included in order to provide a more comprehensive view of that substance (Benzie, 1996a). However, it must be noted that all currently available tests suffer the same drawbacks of being *in vitro*, non-physiological tests. The benefits or biological effect of increased antioxidant intake can only be truly evaluated by monitoring physiological, biochemical or molecular changes which can be shown to represent a decrease in susceptibility to disease, i.e. to assess *in vivo* changes and changes in validated functional markers of health status (Benzie, 1999).

The argument that increased antioxidant defence is protective against ROS-induced damage is compelling. The evidence is so strong that the World Cancer Research Fund recommends that five or more portions of fruits and vegetables should be taken per day, and estimates that 40% of cancers are

preventable by improved diet, mainly involving fruit and vegetable intake (World Cancer Research Fund, 1997). It is believed that the benefits of a fruit and vegetable-rich diet are mediated at least in part through their high antioxidant content. If it is established that increased antioxidant status is indeed beneficial, then realistic dietary strategies to achieve this are needed. Most individuals do not take five or more portions of fruits and vegetables per day, even though this is recommended. If the antioxidant capacity of fruits and vegetables, and other dietary agent such as teas, wines etc., is objectively assessed, and the bioavailability of the antioxidants investigated and established, then an efficient, cost effective and achievable dietary target can be formulated. The FRAP and FRASC assays offer useful tools for the purposes of initial assessment and comparison of *in vitro* antioxidant power and bioavailability of antioxidants in foods.

In this study, some of the questions posed at the beginning have been answered. However, more questions have arisen and are awaiting an answer. Nevertheless, the results of this study will be useful in future studies in this important area. To conclude, the antioxidant defence system is complex and interlinked. Individual components alone may show an antioxidant or pro-oxidant effect in different environments. Every individual member in the system possesses different properties and plays a different role. Co-operative interaction between antioxidants is required for the optimal performance of overall antioxidant defence. Any disturbance of the defence mechanism may lead to adverse effects, i.e. development of oxidative stress-related disease. Enhancing the ability of the body to oppose oxidative stress may resolve or defer the development of oxidative

stress-related disease. Further knowledge on the content and availability of individual antioxidants in foods and their interaction is needed and will help in the strategic planning for the maintenance and prolongation of healthy life through informed food choice.

Table 8.1 Summary of tea catechins absorption studies in human

Measurements	Substance Ingested	Time of Maximal Response	Sampling (blood)	Sampling (urine)	Sample Type Tested	References
EGCG, TRAP, Asc, Glutathione, α -TOC, β -carotene	Greenselect (free form) Phytosome (phospholipid complex form) Equivalent to 400mg EGCG	2 hr for EGCG 3 hr for TRAP (Greenselect Phytosome) 4 hr for TRAP (Greenselect)	0, 1, 2, 3, 4, 5, 6 hr	-	Plasma	Pietta <i>et al.</i> , 1998b
EGCG EGC EC	Decaffeinated green tea solids in 500 ml, with sucrose, coffee whitener, vanilla flavor each gram: 73mg EGCG 68mg EGC 22mg ECG 25mg EC	1.6-2.7hr for EGCG 1.3-1.8 hr for EGC 1.4-1.8 hr for EC ECG not measured	0, 0.5, 2, 4, 8, 24 hr	0-2, 2-6, 6-8, 8-24, 24-48 hr	Plasma	Yang <i>et al.</i> , 1998
Catechins & metabolites, TRAP	overnight fasting 300ml green tea (about 400mg catechins) non-fasting afterwards	EGCG, ECG 2 hr in plasma EGCG, ECG not detected in urine	0, 1, 2, 3, 4, 5 hr	0-6, 6-24, 24-36, 36-48 hr	Plasma	Pietta <i>et al.</i> , 1998a

Table 8.1 Cont'd

Measurements	Substance Ingested	Time of Maximal Response	Sampling (blood)	Sampling (urine)	Sample Type Tested	References
Total catechin	600ml green tea 500ml black tea + 100 ml milk	2 hr	0, 0.5, 1, 2, 4, 6, 8 hr	-	Whole blood	van het Hof <i>et al.</i> , 1998
EGC, EC, EGCG, ECG	non-fasting afterwards 1.2g green tea solid in 200ml water 88mg EGCG 82mg EGC 33mg ECG 32mg EC after breakfast, fasting afterwards	Blood: EGC, EC 1 hr >> 4 hr EGCG 4hr > 1hr ECG Not detected	0, 1, 4 hr	0-3, 3-6, 6-9, 9-12, 12-18, 18-24	Plasma	Lee <i>et al.</i> , 1995
Blood phenols	(+) catechin capsule 92.3 mg/kg weight after breakfast, non-fasting afterwards	12-48 hr Not detected	0-6, 6-12, 12-24, 24-30, 30-36, 36-48, 48-72, 72-96	0-6, 6-12, 12-24, 24-30, 30-36, 36-48, 48-72, 72-96	Whole blood	Das, 1971

Table 8.1 Cont'd

Measurements	Substance Ingested	Time of Maximal Response	Sampling (blood)	Sampling (urine)	Sample Type Tested	References
Total antioxidant power	500ml black tea (1%)	No response	0, 60, 120, 180 min	-	Serum	Maxwell & Thrope, 1996
TRAP	300ml black tea (2%) 300ml green tea (2%)	Black tea 50 min Green tea 30 min	0, 30, 50, 80 min	-	Plasma	Serafini <i>et al.</i> , 1996
	fasting					
FRAP	400ml green tea (5%) fasting	40 min	0, 20, 40, 60, 120 min	0, 30, 60, 90, 120, 150, 180 min	Plasma	Benzie <i>et al.</i> , 1999

Table 8.2 Summary of quercetin absorption studies in human

Measurement	Substance Ingested	Time of Maximal Response	Sampling (blood)	Sampling (urine)	Sample Type Tested	References
Quercetin (unchanged & metabolised form)	4g quercetin overnight fasting	Not detected in plasma & urine (<0.1 mg/l)	0, 20, 40, 80, 160, 320, 540 min & 24 h	0-80, 80-160, 160-320, 320-540 min, 540 min – 24 hr	Plasma	Gugler <i>et al.</i> , 1975
Quercetin	333g onion in breakfast after 3 day quercetin free diet	2.9 h	0, 1, 2, 3, 4, 8, 12, 24, 36, 48 h		Plasma	Hollman <i>et al.</i> , 1996
Quercetin glucoside	300g onion in breakfast after 5 day quercetin free diet	Quercetin glucoside 1.3 h	0, 0.5, 1.0, 1.5, 2, 3, 4, 5, 24 h	0-24 h	Plasma	Aziz <i>et al.</i> , 1998
Isorhamnetin glucoside		Isorhamnetin glucoside 1.8 h				
Quercetin, Kaempferol, Theirs conjugates	1600ml x 3d black tea, = 49mg quercetin x 3 & 27mg kaempferol x 3 129g x 3d fried onions =13mg x 3				Plasma, urine	De Vries <i>et al.</i> , 1998
	low quercetin diet					
TEAC, FRAP, Quercetin, TBARS, SOD, GSH-Px, GR, CAT,	Black currant & apple juice (1:1) 750 ml 1000 ml 1500 ml overnight fasting, then low flavonoid-containing diet		Morning of days 1, 2, 3, 5, 8	24 hr urine	Plasma, Red cells for enzymes	Yong <i>et al.</i> , 1999

Table 8.3 Summary of wine antioxidants absorption studies in human

Measurement	Substance Ingested	Time of Maximal Response	Sampling (blood)	Sampling (urine)	Sample Type Tested	References
TRAP, Total Phenolics, Lutein, zeaxanthin, Lycopene, β - cryptoxanthin, α - & β - carotene, α -tocopherol, AsC, Uric acid	113ml water 113ml dealcoholised white wine 113ml dealcoholised red wine eqv. 300ml wine	50 min for total phenolics & TRAP in red wine	0, 20, 50, 120 min		Plasma	Serafini <i>et al.</i> , 1998
Total phenolics, FRAP, Alcohol	100ml red wine 100ml old malt whisky 100ml new make whisky	30 min for total phenolics & FRAP in red wine & old malt whisky	0, 30, 60, 240, 480 min	4 hr	Plasma	Duthie <i>et al.</i> , 1998

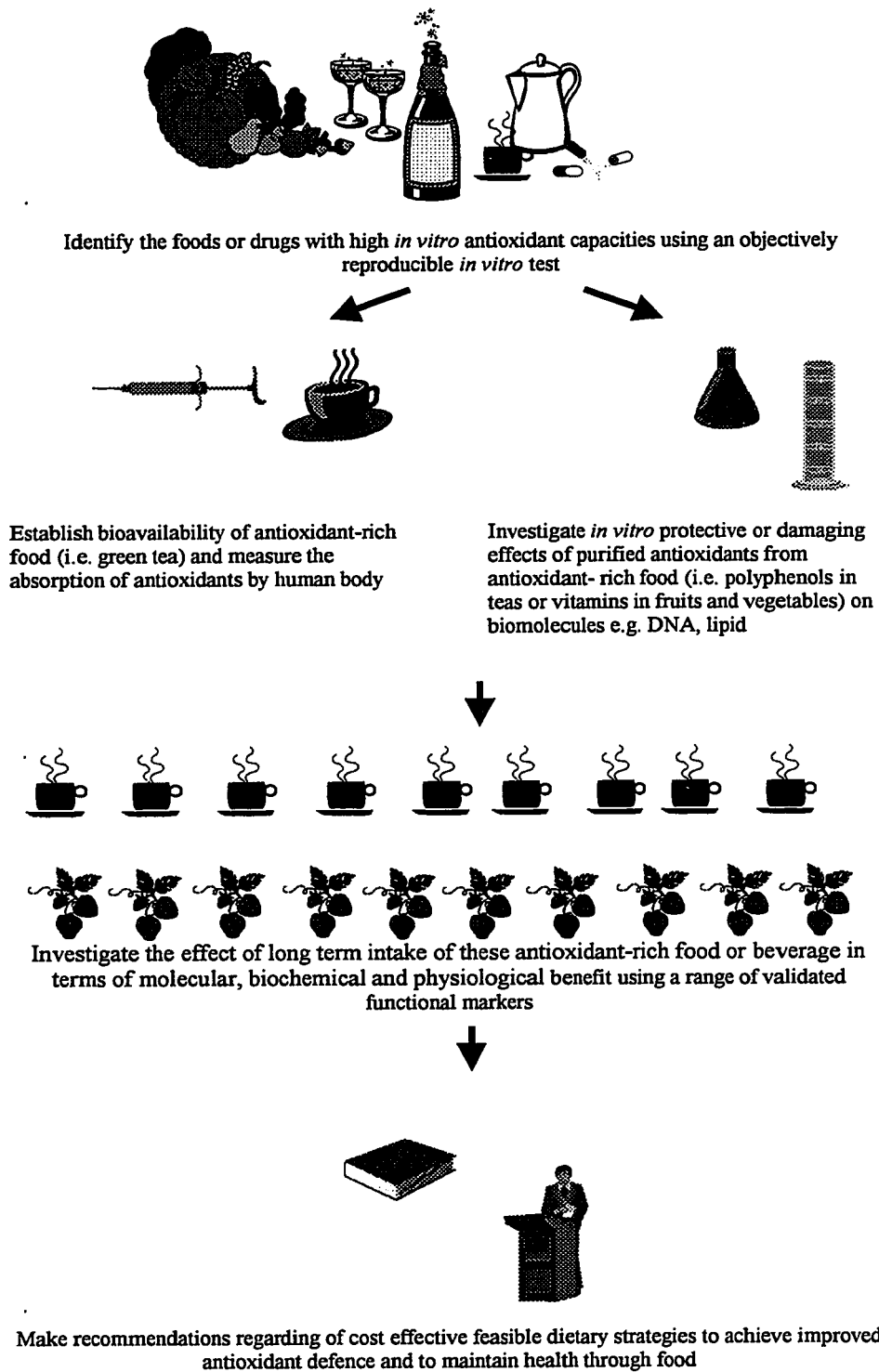


Figure 8.1 Suggested strategy for improving *in vivo* antioxidant status and assessing/achieving health-related benefits



Figure 8.2 No single test can give a complete picture of antioxidant capacity

REFERENCES

- Abu-Amsha, R., Croft, K.D., Puddey, I.B., Proudfoot, J.M., Beilin, L.J. Phenolic content of various beverages determines the extent of inhibition of human serum and low-density lipoprotein oxidation *in vitro*: identification and mechanism of action of some cinnamic acid derivatives from red wine. *Clinical Science* (1996), 91 (4), 449-58.
- Adler, A.J., Holub, B.J. Effect of garlic and fish-oil supplementation on serum lipid and lipoprotein concentrations in hypercholesterolemic men. *American Journal in Clinical Nutrition* (1997), 65 (2), 445-450.
- Afanas'ev, I.B., Dorozhko, A.I., Brodskii, A.V., Kostyuk, V.A., Potapovitch, A.I. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochemical Pharmacology* (1989), 38 (11), 1763-1769.
- Ahlskog, J.E. Parkinson's disease: new treatment strategies. *Comprehensive Therapy* (1990), 16 (12), 41-46.
- Albanes, D., Heinonen, O.P., Huttunen, J.K., Taylor, P.R., Virtamo, J., Edwards, B.K., Haapakoski, J., Rautalahti, M., Hartman, A.M., Palmgren, J. Effects of alpha-tocopherol and beta-carotene supplements on cancer prevention study. *American Journal of Clinical Nutrition* (1995), 62, (6 suppl), 1427S-1430S.
- Albanes, D., Heinonen, O.P., Taylor, P.R., Virtamo, J., Edwards, B.K., Rautalahti, M., Hartman, A.M., Palmgren, J., Freeman, L.S., Haapakoski, J., Barrett, M.J., Pietinen, P., Malila, N., Tala, E., Liippo, K., Salomaa, E.R., Tangrea, J.A., Teppo, L., Askin, F.B., Taskinen, E., Erozan, Y., Greenwald, P., Huttunen, J.K. Alpha-tocopherol and beta-carotene supplements and lung cancer incidence in the alpha tocopherol, beta-carotene cancer prevention study: effects of baseline characteristics and study compliance. *Journal of the National Cancer Institute* (1996), 88, (21), 1560-1570.
- Albano, E., Tomas, A., Parola, M., Comoglio, A., Ingelman-Sundberg, M., Dianzani, M.U. Mechanisms responsible for free radical formation during ethanol metabolism and their role in causing oxidative injury by alcohol. In: Corongiu, F., Banni, S., Dessi, M.A., Rice-Evans, C. eds. *Free Radicals and Antioxidants in Nutrition*. London: Richelieu, 1993.
- Alessio, M.A. Exercise-induced oxidative stress. *Medicine and Science in Sports and Exercise* (1993), 25 (2), 218-224.
- Ambrosone, C.B., Graham, S., Marshall, J.R., Hellmann, R., Nemoto, T., Freudenheim, J.L. Dietary antioxidants and breast cancer risk: effect modification by family history. In: Armstrong, D. ed. *Free Radicals in Diagnostic Medicine*. New York: Plenum Press, 1994, pp 439-440.
- Ames, B.N. Measuring oxidative damage in humans: relation to cancer and ageing. *LARC Scientific Publications* (1988), (89), 407-416.
- Ames, B.N., Shigenaga, M.K., Hagen, T.M. Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the United States of America* (1993), 90 (17), 7915-7922.

Ames, B.N., Cathcart, R., Schwiers, E., Hochstein, P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proceedings of the National Academy of Sciences of the United States of America* (1981), 78 (11), 6858-6862.

Anderson, D., Yu, T.W., Schmezer P. An investigation of the DNA-damaging ability of benzene and its metabolites in human lymphocytes, using the comet assay. *Environmental and Molecular Mutagenesis*. (1995), 26 (4), 305-314.

Araujo, V., Arnal, C., Boronat, M., Ruiz, E., Dominguez, C. Oxidant-antioxidant imbalance in blood of children with juvenile rheumatoid arthritis. *Biofactors* (1998), 8 (1-2), 155-159.

Aruoma, O.I. Characterization of drugs as antioxidant prophylactics. *Free Radical Biology & Medicine* (1995), 20 (5), 675-705.

Aruoma, O.I., Halliwell, B., Aeschbach, R., Löliger, J. Antioxidant and pro-oxidant properties of active rosemary constituents: carnosol and carnosic acid. *Xenobiotica* (1992), 22 (2), 257-268.

Aruoma, O.I., Spencer, J.P.E., Rossi, R., Aeschbach, R., Khan, A., Mahmood, N., Munoz, A., Murcia, A., Butler, J., Halliwell, B. An evaluation of the antioxidant and antiviral action of extracts of rosemary and provencal herbs. *Food and Chemical Toxicology* (1996), 34 (5), 449-456.

Aziz, A.A., Edwards, C.A., Lean, M.E. Crozier, A. Absorption and excretion of conjugated flavonols, including quercetin-4'-O-beta-glucoside and isorhamnetin-4'-O-beta-glucoside by human volunteers after the consumption of onions. *Free Radical Research* (1998), 29 (3), 257-269.

Balentine, D.A. Manufacturing and chemistry of tea. In: Tang H.C., Chang, Y.L., Tuan, H.H. eds. *Phenolic Compounds in Food and their Effects of Health Vol 1*. Washington, DC: American Chemical Society, 1992, pp 102-17.

Baron, J.A., Gerhardsson de Verdier, M., Ekbom, A. Coffee, tea, tobacco, and cancer of the large bowel. *Cancer Epidemiology, Biomarkers & Prevention* (1994), 3 (7), 565-570.

Bendich, A., Machlin, L.J., Scandurra, O. The antioxidant role of vitamin C. *Advances in Free Radical Biology and Medicine* (1986), 2, 419-444.

Benzie, I.F.F. Lipid peroxidation: a review of causes, consequences, measurement and dietary influences. *International Journal of Food Science and Nutrition* (1996a), 47, 233-261.

Benzie, I.F.F. An automated, specific, spectrophotometric method for measuring ascorbic acid in plasma (EFTSA). *Clinical Biochemistry* (1996b), 29 (2), 111-116.

Benzie I.F.F. Antioxidants: observational epidemiology. In: The Encyclopedia of Nutrition. Sadler, M.J., Strain, J.J., Cabellero, B. eds. London: Academic Press, 1998, pp 106-115.

Benzie, I.F.F. Vitamin C: prospective functional markers for defining optimal nutritional status. *Proceedings of the Nutrition Society* (1999), 58, 1-8.

Benzie, I.F.F., Chung, W.Y. Total antioxidant power of plasma: male-female differences and effect of anticoagulant used. *Annals of Clinical Biochemistry* (1999), 36, 104-106.

Benzie, I.F.F., Strain, J.J. The effect of ascorbic acid on the measurement of total cholesterol and triglycerides: possible artefactual lowering in individuals with high plasma concentration of ascorbic acid. *Clinica Chimica Acta* (1995), 239 (2), 185-190.

Benzie, I.F.F., Strain, J.J. Uric acid: friend or foe? *Redox Report* (1996a), 2 (4), 231-234.

Benzie, I.F.F., Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': The FRAP Assay. *Analytical Biochemistry* (1996b), 239 (1), 70-76.

Benzie, I.F.F., Strain, J.J. Acute Post-ingestion changes in plasma ascorbic acid concentration relationship to dose and to existing body stores. *Nutrition Research* (1997a), 17 (2), 187-190.

Benzie, I.F.F., Strain, J.J. Simultaneous automated measurement of total 'antioxidant' (reducing capacity and ascorbic acid concentration. *Redox Report* (1997b), 3 (4), 233-238.

Benzie, I.F.F., Strain, J.J. Ferric reducing / antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. In: Packer, L. ed. *Methods in Enzymology Vol 299*. Orlando: Academic Press, 1999, pp 15-27.

Benzie, I.F.F., Szeto, Y.T. Total antioxidant capacity of teas by the ferric reducing / antioxidant power (FRAP) assay. *Journal of Agricultural and Food Chemistry* (1999), 47 (2), 633-636.

Benzie, I.F.F., Szeto, Y.T., Strain, J.J., Tomlinson, B. Consumption of green causes rapid increase in plasma antioxidant power in humans. *Nutrition and Cancer* (1999), 34 (1), 83-87.

Benzie, I.F.F., Janus E.D., Strain J.J. Plasma vitamin C and E levels in Hong Kong Chinese. *European Journal of Clinical Nutrition* (1998), 52 (6), 447-451.

Benzie, I.F.F., Tomlinson, B. Antioxidant power of angiotensin-converting inhibitors *in vitro*. *British Journal of Clinical Pharmacology* (1998), 45 (2), 168-169.

Bhagavan, N.V. *Medical Biochemistry*. London: Jones and Bartlett, 1991.

Bingham, S.A. Vorster, J.C., Jerling, J.C. Magee, E., Mulligan, A., Runswick, S.A., Cummings, J.H. Effect of black tea drinking on blood lipids, blood pressure and aspects of bowel habit. *British Journal of Nutrition* (1997), 78 (1), 41-45.

Blot, W.J., McLaughlin, J.K., Chow, W.H. Cancer rates among drinkers of black tea. *Critical Reviews in Food Science & Nutrition* (1997), 37 (8), 739-760.

Bode, A.M. Metabolism of vitamin C in health and disease. In: Sies, H. ed. *Antioxidants in Disease Mechanisms and Therapy, Advances in Pharmacology, Vol 38*. London: Academic Press, 1997, pp 21-47.

Borenstein, B. The role of ascorbic acid in foods. *Food Technology* (1987), 41 (11), 98-99.

Bors, W., Heller, W., Michel, C., Stettmaier, K. Flavonoids and polyphenols: chemistry and biology. In: Cadenas, E., Packer, L. eds. *Handbook of Antioxidants*. New York: Marcel Dekker, Inc., 1996, pp 21-47.

Brinkley, L.J., Gregory, J., Pak, C.Y. A further study of oxalate bioavailability in foods. *Journal of Urology* (1990), 144 (1), 94-96.

Brinkley, L.J., McGuire, J., Gregory, J., Pak, C.Y. Bioavailability of oxalate in foods. *Urology* (1981), 17 (6), 534-538.

British Pharmacopoeia Commission. *British Pharmacopoeia Vol 1*. London: HMSO, 1998.

Buettner, G.R. The pecking order of free radicals and antioxidants: lipid peroxidation, α -tocopherol, and ascorbate. *Archives of Biochemistry & Biophysics* (1992), 300 (2), 535-543.

Bunker, V.W. Free radicals, antioxidants and ageing. *Medical Laboratory Science* (1992), 49, 299-312.

Cadenas, E. Mechanisms of oxygen activation and reactive oxygen species detoxification. In: Ahmad, S. ed. *Oxidative Stress and Antioxidant Defenses in Biology*. New York: Chapman & Hall, 1995, pp 1-61.

Cadenas, E., Packer, L. eds. *Handbook of Antioxidants*. New York: Marcel Dekker, Inc., 1996.

Campos, A.M., Lissi, E.A. Total antioxidant potential of Chilean wines. *Nutrition Research* (1996), 16 (3), 385-389.

Cao, G., Alessio, H.M., Cutler, R.G. Oxygen radical absorbance capacity assay for antioxidants. *Free Radical Biology & Medicine* (1993), 14 (3), 303-311.

Cao, G., Prior, R.L. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clinical Chemistry* (1998), 44 (6), 1309-1315.

Cao, G., Sofic, E., Prior, R.L. Antioxidant capacity of tea and common vegetables. *Journal of Agricultural and Food Chemistry* (1996), 44 (11), 3426-3431.

Cao, G., Verdon, C.P., Wu, A.H.B., Wang, H., Prior, R.L. Automated assay of oxygen radical absorbance capacity with COBAS FARA. *Clinical Chemistry* (1995), 41 (12), 1738-1744.

Cao, G., Sofic, E., Prior, R.L. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radical Biology & Medicine*. (1997), 22 (5), 749-60.

Chen, Y., Miles, A.M., Grisham, M.B. Pathophysiology and reactive oxygen metabolites. In: Ahmad, S. ed. *Oxidative Stress and Antioxidant Defenses in Biology*. New York: Chapman & Hall, 1995, pp 62-95.

Collins, A.R., Dusinska, M., Franklin, M., Somorovská, M., Petrovská, H., Duthie, S., Fillion, L., Panayiotidis, M., Rašlová, K., Vaughan, N. Comet assay in human biomonitoring studies: reliability, validation, and applications. *Environmental and Molecular Mutagenesis* (1997), 30, 139-146.

Collins, A.R., Dusinska, M., Gedik, C.M., Stetina, R. Oxidative damage to DNA: do we have a reliable biomarker? *Environmental Health Perspectives* (1996), 104 (Suppl 3), 465-469.

Combs, G.F. *The Vitamins: Fundamental Aspects in Nutrition and Health*. London: Academic Press, 1992.

Comstock, G.W., Burke, A.E., Hoffman, S.C., Helzlsouer, K.J., Bendich, A., Masi, A.T., Norkus, E.P., Malamet, R.L., Gershwin, M.E. Serum concentrations of alpha tocopherol, beta carotene, and retinol preceding the diagnosis of rheumatoid arthritis and systemic lupus erythematosus. *Annals of the Rheumatic Diseases* (1997), 56 (5), 323-325.

Cook, N., Samman, S. Flavonoids – chemistry, metabolism, cardioprotective effects, and dietary sources. *Nutritional Biochemistry* (1996), 7, 66-76.

Cooke, M. Podmore, I., Herbert, K., Lunec, J., Thomas, S. UV-mediated DNA damage and its assessment. *Toxicology and Ecotoxicology News* (1996), 3 (4), 101-109.

Coudray, C., Richard, M.J., Favier, A.E. Determination of primary and secondary lipid peroxidation products: plasma lipid hydroperoxides and thiobarbituric acid reactive substances. In: Favier, A.E., Cadet, J., Kalyanaraman, B., Fontecave, M., Pierre, J.L. eds. *Analysis of Free Radicals in Biological Systems*. Switzerland: Birkhauser, 1995, pp 185-200.

Cutler, R.G. Antioxidants and aging. *American Journal of Clinical Nutrition* (1991), 53 (1 Suppl), 373S-379S.

Das, J.R., Bhat, S.G., Gowda, L.R. Purification and characterization of a polyphenol oxidase from the Kew cultivar of Indian pineapple fruit. *Journal of Agricultural and Food Chemistry* (1997), 45 (6), 2031-2035.

Das, N.P., Studies on flavonoid metabolism. *Biochemical Pharmacology* (1971), 20 (12), 3435-3445.

Davies, K.J.A. Oxidative Stress: the paradox of aerobic life. In: Rice-Evans, C. Halliwell, B., Lunt, G.G. eds. *Biochemical Society Symposium No 61, Free Radicals and Oxidative Stress: Environment, Drugs and Food Additives*. London: Portland Press, 1995, pp. 1-31.

Davies, K.J.A., Sevanian, A. Musikkassah-Kelly, S.F., Hochstein, P. Uric acid-iron ion complexes: a new aspect of the antioxidant functions of uric acid. *Biochemical Journal* (1986), 235 (3), 747-754.

Day, A., Stansbie, D. Cardioprotective effect of red wine may be mediated by urate. *Clinical Chemistry* (1995), 41 (9), 1319-1320.

Day, A.P., Kemp, H.J., Bolton, C., Hartog, M., Stansbie, D. Effect of concentrated red grape juice consumption on serum antioxidant capacity and low-density lipoprotein oxidation. *Annals of Nutrition & Metabolism* (1997), 41 (6), 353-357.

Department of Health. Dietary reference values for food energy and nutrients for the United Kingdom. *Dep. Hlth Soc. Subj. No 41*. London: DHSS, 1991.

Desfaits, A.C., Serri, O., Renier, G. Normalization of plasma lipid peroxides, monocyte adhesion, and tumor necrosis factor-alpha production in NIDDM patients after gliclazide treatment. *Diabetes Care* (1998), 21 (4), 487-493.

Diplock, A.T. Vitamin E. In: Diplock, A.T. ed. *Fat Soluble Vitamins: Their Biochemistry And Applications*. London: Heinemann, 1985, pp 154-224.

Diplock, A.T. Antioxidants and disease prevention. *Molecular Aspects of Medicine* (1994), 15 (4), 293-376.

Doll, R., Peto, R., Hall, E., Wheatley, K., Gray, R. Mortality in relation to consumption of alcohol: 13 years' observation on male British doctors. *British Medical Journal* (1994), 309 (6959), 911-918.

Donnelly, J.K., Robinson, D.S. Free radicals in foods. *Free Radical Research* (1995), 22 (2), 147-176.

Dreosti I.E., Wargovich M.J., Yang C.S. Inhibition of carcinogenesis by tea: the evidence from experimental studies. *Critical Reviews in Food Science & Nutrition* (1997), 37 (8), 761-70.

Duthie, G.G., Pedersen, M.W., Gardner, P.T., Morrice, P.C., McE Jenkinson, A., McPhail D.B., Steele, G.M. The effect of whisky and wine consumption on total phenol content and antioxidant capacity of plasma from healthy volunteers. *European Journal of Clinical Nutrition* (1008), 52, 733-736.

Duthie, S.J., Collins, A.R., Duthie G.G., Dobson, V.L. Quercetin and myricetin protect against hydrogen peroxide-induced DNA damage (strand breaks and oxidised pyrimidines) in human lymphocytes. *Mutation Research* (1997), 393 (3), 223-231.

Ebadi, M., Srinivasan, S.K., Baxi, M.D. Oxidative stress and antioxidant therapy in Parkinson Disease. *Progress in Neurobiology* (1996), 48 (1), 1-19.

Ebenbichler, C.F., Kirchmair, R., Egger, C. Patch, J.R. Postprandial state and atherosclerosis. *Current Opinion in Lipidology* (1995), 6 (5), 286-290.

Emerit, I. Reactive oxygen species, chromosome mutation and cancer: possible role of clastogenic factors in carcinogenesis. *Free Radical Biology and Medicine* (1994), 16 (1), 99-109.

Epe, B. DNA damage induced by photosensitization. In: Halliwell, B., Aruoma, O.I. eds. *DNA and Free Radicals*. New York: Ellis-Horwood, 1993, pp 41-65.

Eriksson, C.E., Na, A. Antioxidant agents in raw materials and processed foods. In: Corongiu, F. Banni, S. Dessi, M.A., Rice-Evans, C. eds. *Free Radicals and Antioxidants in Nutrition*. London: Richelieu, 1993, pp 205-224.

Esterbauer, H., Striegl, G. Puhl, H., Rotheneder, M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radical Research & Communication* (1989), 6 (1), 67-75.

Everett, S.A., Dennis, M.F., Patel, K.B., Maddix, S., Kundu, S.C., Willson, R.L. Scavenging of nitrogen dioxide, thiyl, and sulfonyl free radicals by the nutritional antioxidant β -carotene. *Journal of Biological Chemistry* (1996), 271 (8), 3988-3994.

Ferrari, R. Oxygen-free radicals at myocardial level: effect of ischaemia and reperfusion. In: Armstrong, D. ed. *Free Radicals in Diagnostic Medicine*. New York: Plenum Press, 1994, pp 99-128

- Finch, A.M., Kasidas, G.P., Rose, G.A. Urine composition in normal subjects after oral ingestion of oxalate-rich foods. *Clinical Science* (1981), 60 (4), 411-418.
- Fischer, V., West, P.R., Harman, L.S., Mason, R.P. Free radical metabolites of acetaminophen and a dimethylated derivative. *Environmental Health Perspectives* (1985), 64, 127-137.
- Frankel, E.N., Kanner, J., German, J.B., Parks, E., Kinsella, K.E. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* (1993), 314 (8843), 454-457.
- Fruchart, J.C., Duriez, P. Free radicals and atherosclerosis. In: Rice-Evans, C.A., Burdon, R.H. eds. *Free Radical Damage and Its Control*. New York: Elsevier Science, 1994, pp 257-280.
- Fukushima, S., Takada, N., Hori, T., Wanibuchi, H., Cancer prevention by organosulfur compounds from garlic and onion. *Journal of Cellular Biochemistry - Supplement*, (1997), 27, 100-105.
- Gale, C.R., Martyn, C.N., Winter, P.D., Cooper, C. Vitamin C and risk of death from stroke and coronary heart disease in cohort of elderly people. *British Medical Journal* (1995), 310 (6994), 1563-1566.
- Gambhir, J.K., Lali, P., Jain, A.K. Correlation between blood antioxidant levels and lipid peroxidation in rheumatoid arthritis. *Clinical Biochemistry* (1997), 30 (4), 351-355.
- Gao, Y.T., McLaughlin, J.K., Blot, W.J., Ji, B.T., Dai, Q., Fraumeni, J.F. Reduced risk of Esophageal cancer associated with green tea consumption. *Journal of the National Cancer Institute* (1994), 86 (11), 855-858.
- Garrel, C., Fontecave, M. Nitric oxide: chemistry and biology. In: Favier, A.E., Cadet, J., Kalyanaraman, B., Fontecave, M., Pierre, J.L. eds. *Analysis of Free Radicals in Biological Systems*. Switzerland: Birkhauser, 1995, pp 21-35.
- Gey, K.F. Prospects for the prevention of free radical disease, regarding cancer and cardiovascular disease. *British Medical Bulletin* (1993), 49 (3), 679-699.
- Gey, K.F. Ten-year retrospective on the antioxidant hypothesis of arteriosclerosis: threshold plasma levels of antioxidant micronutrients related to minimum cardiovascular risk. *Nutritional Biochemistry* (1995), 6, 206-236.
- Gey, K.F. Vitamins E plus C and interacting conutrients required for optimal health. A critical and constructive review of epidemiology and supplementation data regarding cardiovascular disease and cancer. *Biofactors* (1998), 7 (1-2), 113-74.
- Gey, K.F., Puska, P., Jordan, P., Moser, U.K. Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. *American Journal of Clinical Nutrition* (1991), 53 (1 Suppl), 326S-334S.
- Ghiselli, A., Serafini, M., Maiani, G., Azzini, E., Ferro-Luzzi, A. A fluorescence-based method for measuring total plasma antioxidant capability. *Free Radical Biology and Medicine* (1995), 18 (1), 29-36.
- Ginter, E. Ascorbic acid in cholesterol metabolism and in detoxification of xenobiotic substances: problem of optimum vitamin C intake. *Nutrition* (1989), 5 (6), 369-374.

Goldberg, D.M. Dose wine work? (editorial) *Clinical Chemistry* (1995), 41 (1), 14-16.

Goldbohm, R.A., Hertog, M.G., Brants, H.A.M., van Poppel, G., van den Brandt, P.A. Consumption of black tea and cancer risk: a prospective cohort study. *Journal of the National Cancer Institute* (1996), 88 (2), 93-100.

de Graff, J., Hak-Lemmers, H.L., Hectors, M.P. Demacker, P.N., Hendriks, J.C., Stalenhoef, A.F. Enhanced susceptibility to *in vitro* oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arteriosclerosis and Thrombosis* (1991), 11 (2), 298-306.

Graham, H.N. Green tea composition, consumption, and polyphenol chemistry. *Preventive Medicine* (1992), 21 (3), 334-350.

Granger, D.N., Rutili, G., McCord, J.M. Superoxide radicals in feline intestinal ischemia. *Gastroenterology* (1981), 81 (1), 22-29.

Greenberg, E.R., Baron, J.A., Karagas, M.R., Stukel, T.A., Nierenberg, D.W., Stevens, M.M., Mandel, J.S., Haile, R.W. Mortality associated with low plasma concentration of beta carotene and the effect of oral supplementation. *Journal of American Medical Association* (1996), 275 (9), 699-703.

Gronbaek, M., Deis, A., Sorensen, T.I.A., Becker, U., Schnohr, P., Jensen G. Mortality associated with moderate intakes of wine, beer, or spirits. *British Medical Journal* (1995), 310 (6988), 1165-1169.

Gugler, R., Leschik, M., Dengler, H.K. Disposition of quercetin in man after single oral and intravenous dose. *European Journal of Clinical Pharmacology* (1975), 9 (2-3), 229-234.

Gutteridge, J.M.C., Halliwell, B. *Antioxidants in Nutrition, Health, and Disease*. New York: Oxford University Press, 1994.

Halliwell, B. The role of oxygen radicals in human disease, with particular reference to the vascular system. *Haemostasis* (1993), 23 (suppl 1), 118-126.

Halliwell, B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence?. *Lancet* (1994), 344 (8924), 721-724.

Halliwell, B. The biological significance of oxygen-derived species. In: Valentine, J.S., Foote, C.S., Grennberg, A., Liebman, J.F. eds. *Active Oxygen in Biochemistry*. Glasgow: Blackie Academic & Professional, 1995a, pp 313-335.

Halliwell, B. How to characterize an antioxidant: an update. In: Rice-Evans, C. Halliwell, B., Lunt, G.G. ed. *Biochemical Society Symposium No 61, Free Radicals and Oxidative Stress: Environment, Drugs and Food Additives*. London: Portland Press, 1995b, pp 73-101.

Halliwell, B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in human. *Free Radical Research* (1996), 25 (1), 57-74.

Halliwell, B. Antioxidants: The basics-what they are and how to evaluate them. In: Sies, H. ed. *Antioxidants in Disease Mechanisms and Therapy, Advances in Pharmacology, Vol 38*. London: Academic Press, 1997, pp 3-20.

Halliwell, B., Gutteridge J.M.C. eds. *Free Radicals in Biology and Medicine 2nd Ed.*: Oxford: Clarendon Press, 1989.

Halliwell, B., Gutteridge J.M.C. eds. *Free Radicals in Biology and Medicine 3rd Ed.*: Oxford: Clarendon Press, 1999.

Halliwell, B., Murcia, M.A., Chirico, S., Aruoma, O.I. Free radical and antioxidants in food and *in vivo*: what they do and how they work. *Critical Reviews in Food Science Nutrition* (1995), 35 (1&2), 7-20.

Hamilton-Miller J.M. Antimicrobial properties of tea (*Camellia sinensis* L.). *Antimicrobial Agents & Chemotherapy* (1995), 39 (11), 2375-2377.

Hansson, L.E., Nyren, O., Bergstrom, R., Alicja, W., Lindgren, A., Baron, J., Adami, H.O. Diet and risk of gastric cancer. A population-based case-control study in Sweden. *International Journal of Cancer* (1993), 55 (2), 181-189.

Hasegawa, R., Chujo, T., Sai-kato, K., Umemura, T., Tanmura, A., Kurokawa, Y. Preventive effects of green tea against liver oxidative DNA damage and hepatotoxicity in rats treated with 2-nitropropane. *Food and Chemical Toxicology* (1995), 33 (11), 961-970.

Helrich, K. ed., Official Methods of Analysis of AOAC International, 16th Ed. Arlington: AOAC International, 1995.

Hennekens, C.H., Buring, J.E., Manson, J.E., Stampfer, M., Rosner, B., Cook, N.R., Belanger, C., LaMotte, F., Gaziano, J.M., Ridker, P.M., Willett, W., Peto, R. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasma and cardiovascular disease. *The New England of Medicine* (1996), 334 (18), 1145-1149.

Herrmann, K. Flavonols and flavones in food plants: a review. *Journal of Food Technology* (1976), 11, 433-448.

Herrmann, K. Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *Critical Reviews in Food Science & Nutrition* (1989), 28 (4), 315-347.

Hertog, M.G.L., Hollman, P.C.H., van de Putte, B. Content of potentially anticarcinogenic flavonoids of tea infusions, wines and fruit juices. *Journal of Agricultural Food Chemistry* (1993), 41 (8), 1242-1246.

Hertog, M.G., Sweetnam, P.M., Fehily, A.M., Elwood, P.C., Kromhout, D. Antioxidant flavonols and ischemic heart disease in a Welsh population of men: the Caerphilly study. *American Journal of Clinical Nutrition* (1997), 65 (5), 1489-1494.

Hikino, H. Ishiyama, M. Suzuki, Y., Konno, C. Mechanisms of hypoglycemic activity of Ganoderan B: a glycan of *Ganoderma lucidum* fruit bodies. *Planta Medica* (1989), 55 (5), 423-428.

Hiramatsu, M., Edamatsu, R., Ohyama, H. & Mori, A. Effect of Japanese herbal medicine, sho-saiko-to-go-keishi-kashakuyaku-to (TJ-960) on aging. In: Ong, A.S.H., Packer, L. eds. *Lipid-Soluble Antioxidants Biochemistry and Clinical Applications*. Switzerland: Birkhauser, 1992, pp 535-552.

Ho, R.Y.N., Liebman, J.F., Valentine, J.S. Biological reactions of dioxygen: an introduction. In: Valentine, J.S., Foote, C.S., Grennberg, A., Liebman, J.F. eds. *Active Oxygen in Biochemistry*. Glasgow: Blackie Academic & Professional, 1995, pp 1-36.

Holland, B., Welch, A.A., Unwin, I.D., Buss, D.H., Paul, A.A., Southgate, D.A.T. eds. *McCance and Widdowson's The Composition of Foods*, 5th revised & extended ed, Cambridge: The Royal Society of Chemistry and Ministry of Agriculture, Fisheries and Food, 1991.

Hollman, P.C.H. Bioavailability of flavonoids. *European Journal of Clinical Nutrition* (1997), 51 (Suppl 1), S66-S69.

Hollman, P.C.H., Gaag, M.V.D., Mengelers, M.J.B., Van Trijp, J.M.P., De Vries, J.H.M., Katan, M.B. Absorption and disposition kinetics of the dietary antioxidant quercetin in man. *Free Radical Biology and Medicine* (1996), 21 (5), 703-707.

Hollman, P.C., Katan, M.B. Absorption, metabolism and health effects of dietary flavonoids in man. *Biomedicine & Pharmacotherapy* (1997), 51 (8), 305-10.

Hollman, P.C.H., Tijburg, L.B.M., Yang, C.S. Bioavailability of flavonoids from tea. *Critical Reviews in Food Science and Nutrition* (1997), 37 (8), 719-738.

Imai, J., Ide, N., Nagae, S. Moriguchi, T., Matsuura, H. Itakura, Y. Antioxidants and radical scavenging effects of aged garlic extract and its constituents. *Planta Medica* (1994), 60 (5), 417-420.

Imai, K., Nakachi, K. Cross sectional study of effects of drinking green tea on cardiovascular and liver diseases. *British Medical Journal* (1995), 310 (6981), 693-696.

Inoue, S., Ito, K., Yamamoto, K., Kawanishi, S. Caffeic acid causes metal-dependent damage to cellular and isolated DNA through H₂O₂ formation. *Carcinogenesis* (1992), 13 (9), 1497-1502.

Ip, S.P., Ko, K.M. The crucial antioxidant action of schisandrin B in protecting against carbon tetrachloride hepatotoxicity in mice: a comparative study with butylated hydroxytoluene. *Biochemical Pharmacology* (1996), 52 (11), 1687-1693.

Ishikawa, T., Suzukawa, M., Ito, T., Yoshida, H., Ayaori, M., Nishiwaki, M., Yonemura, A., Hara, Y., Nakamura, H. Effect of tea flavonoid supplementation on the susceptibility of low-density lipoprotein to oxidative modification. *American Journal of Clinical Nutrition* (1997), 66 (2), 261-266.

Ito, N., Hirose, M. Antioxidants – carcinogenic and chemopreventive properties. *Advances in Cancer Research* (1989), 53, 247-302.

Jacques, P.F. Relationship of vitamin C status to cholesterol and blood pressure. *Annals of the New York Academy of Sciences* (1992), 669, 205-213.

Jenner, P. Dexter, D.T., Sian, J., Schapira, A.H., Marsden, C.D. Oxidative stress as a cause of nigral cell death in Parkinson's disease and incidental Lewy body disease. *Annals of Neurology* (1992), 32 Suppl., S82-S87.

Kagan, V.E., Nohl, H., Quinn, P.J. Coenzyme Q: its role in scavenging and generation of radicals in membranes. In: Cadenas, E., Packer, L. eds. *Handbook of Antioxidants*. New York: Marcel Dekker, Inc. 1996, pp 157-201.

Kaltwasser, J.P., Werner, E., Schalk, K., Hansen, C., Gottschalk, R., Seidl, C. Clinical trial on the effect of regular tea drinking on iron accumulation in genetic haemochromatosis. *Gut* (1998), 43 (5), 699-704.

Kanner, J., Frankel, E., Granit, R., German, B., Kinsella, J.E. Natural antioxidants in grapes and wines. *Journal of Agricultural Food Chemistry* (1994), 42 (1), 64-69.

Kaplan, A., Jack, R., Opheim, K.E., Toivola, B., Lyon, A.W. eds. *Clinical Chemistry: Interpretation and Techniques*. 4th ed. Malvern: Williams & Wilkins, 1995.

Kaul, N., Siveski-Iliskovic, N., Hill, M., Khaper, N., Seneviratne, C., Singal, P.K. Probucol treatment reverses antioxidant and functional deficit in diabetic cardiomyopathy. *Molecular & Cellular Biochemistry* (1996), 160-161, 283-288.

Kaul, N., Siveski-Iliskovic, N., Thomas, T.P., Hill, M., Khaper, N., Singal, P.K. Probucol improves antioxidant activity and modulates development of diabetic cardiomyopathy. *Nutrition* (1995), 11 (5 Suppl), 551-554.

Kendler, B.S., Free radicals in health and disease: implications for primary health care providers. *Nurse Practitioner* (1995), 20 (7), 29-43.

Kinlen, L.J., Willows, A.N., Oldblatt, P., Yudkin, J. Tea consumption and cancer. *British Journal of Cancer* (1988), 58 (3), 397-401.

Klatsky, A.L., Armstrong, M.A., Friedman, G.D. Coffee, tea, and mortality. *Annals of Epidemiology* (1993), 3 (4), 375-381.

Klaunig, J.E. Chemopreventive effects of green tea components on hepatic carcinogenesis. *Preventive Medicine* (1992), 21 (4), 510-519.

Ko, K.M., Yick, P.K., Poon, M.K.T., Che, C.T. *Schisandra chinensis*-derived antioxidant activities in "Sheng Mai San", a compound formulation, *in vivo* and *in vitro*. *Phytotherapy Research* (1995), 9, 203-206.

Kohlmeier, L. Has the tea been ruined? (guest editorial) *British Journal of Nutrition* (1997), 78 (1), 1-3.

Kohlmeier, L., Weterings, K.G.C., Steck, S., Kok, F.J. Tea and cancer prevention: an evaluation of the epidemiological literature. *Nutrition & Cancer* (1997), 27 (1), 1-13.

Kong, X.L. Tea leaves production, trade and others. In: *Zhongwai Chashi*. Shanghai: Shang-hai Wen Hua Chu Ban She, 1993.

Korytowski, W., Girotti, A.W. Lipid hydroperoxide analysis by reverse-phase high-performance liquid chromatography with mercury cathode electrochemical detection. In: Favier, A.E., Cadet, J., Kalyanaraman, B., Fontecave, M., Pierre, J.L. eds. *Analysis of Free Radicals in Biological Systems*. Switzerland: Birkhauser, 1995, pp 165-183.

Kushi, L.H., Folsom, A.R., Prineas, R.J., Mink, P.J., Wu, Y., Bostick, R.M. Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women. *New England Journal of Medicine* (1996), 334 (18), 1156-1162.

Paganga, G., Al-Hashim, H., Khodr, H., Scott, B.C., Aruoma, O.I., Hider, R.C., Halliwell, B., Rice-Evans, C.A. Mechanisms of antioxidant activities of quercetin and catechin. *Redox Report* (1996), 2 (6), 359-364.

La Vecchia, C., Negri, E., Francheschi, S. D'Avanzo, B., Boyle, P. Tea consumption and cancer risk. *Nutrition & Cancer* (1992), 17 (1), 27-31.

Laakso, M., Lehto, S., Penttilä, I., Pyörälä, K. Cardiovascular risk factors, lipids, and insulin: lipids and lipoproteins predicting coronary heart disease mortality and morbidity in patients with non-insulin-dependent diabetes. *Circulation* (1993), 88 (4), 1421-1430.

Laaksonen, R., Jokelainen, K., Laakso, J., Sahi, T., Harkonen, M., Tikkanen, M.J., Himberg, J.J. The effect of simvastatin treatment on natural antioxidants in low-density lipoproteins and high-energy phosphates and ubiquinone in skeletal muscle. *The American Journal of Cardiology* (1996), 77 (10), 851-854.

Laranjinha, J.A., Almeida, L.M., Madeira, V.M. Reactivity of dietary phenolic acids with peroxyl radicals: antioxidant activity upon low density lipoprotein peroxidation. *Biochemical Pharmacology* (1994), 48 (3), 487-494.

Laughton, M.J., Halliwell, B., Evans, P.J., Houlst, J.R.S. Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin, Effect on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. *Biochemical Pharmacology* (1989), 38 (17), 2859-2865.

Leanderson, P.; Faresjo, A.O.; Tagesson, C. Green tea polyphenols inhibit oxidant-induced DNA strand breakage in cultured lung cells. *Free Radical Biology & Medicine* (1997), 23 (2), 235-242.

Lee, M.J. Wang, Z.Y., Li, H., Chen, L., Sun, Y., Gobbo, S., Balentine, D.A., Yang, C.S. Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiology, Biomarkers & Prevention* (1995), 4 (4), 393-399.

St Leger, A.S., Cochrane, A.L. Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. *Lancet* (1979), 1 (8124), 1017-1020.

Levine, M., Conry-Cantilena, C., Wang Y., Welch, R.W., Washko, P.W., Dhariwal, K.R., Park, J.B., Lazarey, A., Granumlich, J.F., King, J., Cantilena, L.R. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proceedings of the National Academy of Sciences of the United States of America* (1996), 93(8), 3704-3709.

Lin J.M., Lin, C.C., Chen, F.F., Ujiie, T., Takada, A. Radical scavenger and antihepatotoxic activity of *Ganoderma formosanum*, *Ganoderma lucidum* and *Ganoderma neo-japoicum*. *Journal of Ethnopharmacology* (1995), 47 (1), 33-41.

Lin, Y.L., Juan, I.M., Chen, Y.L., Liang, Y.C., Lin, J.K. Composition of polyphenols in fresh tea leaves and associations of their oxygen-radical-absorbing capacity with antiproliferative actions in fibroblast cells. *Journal of Agricultural Food Chemistry* (1996), 44 (6), 1387-1394.

Loop, R.A., Anthony, M., Willis, R.A., Folkers, K. Effects of ethanol, lovastatin and coenzyme Q₁₀ treatment on antioxidants and TBA reactive material in liver of rats. *Molecular Aspects in Medicine* (1994), 15 (Suppl), S195-S206.

Luo, M., Kannar, K., Wahlqvist, M.L., O'Brien, R.C. Inhibition of LDL Oxidation by Green Tea Extract. (comment, letter) *Lancet* (1997), 349 (9048), 360-361.

Lutz, U., Lugli, S., Bitsch, A., Schlatter, J., Lutz, W.K. Dose response for the stimulation of cell division by caffeic acid in forestomach and kidney of the male F344 rat. *Fundamental & Applied Toxicology* (1997), 39 (2), 131-137.

Machlin, L.J. Critical assessment of the epidemiological data concerning the impact of antioxidant nutrients on cancer and cardiovascular disease. *Critical Reviews in Food Science and Nutrition* (1995), 5 (1&2), 41-50.

Macrae, R., Robinson, R.K., Sadler, M.J. eds. *Encyclopaedia of Food Science. Food Technology and Nutrition*. London; San Diego: Academic Press, 1993.

Makrigiorgos, G.M. Detection of lipid peroxidation on erythrocytes using the excimer-forming property of a lipophilic BODIPY fluorescent dye. *Journal of Biochemical & Biophysical Methods* (1997), 35 (1), 23-35.

Mantha, S.V., Kalra, J., Prasad, K. Effects of probucol on hypercholesterolemia-induced changes in antioxidant enzymes. *Life Sciences* (1996), 58 (6), 503-509.

Maples, K.R., Mason, R.P. Free radical metabolite of uric acid. *Journal of Biological Chemistry* (1988), 263 (5), 1709-1712.

Mares-Perlman, J.A., Brady, W.E., Klein, B.E.K., Klein, R., Palta, M., Bowen, P., Stacewicz-Sapuntzakis, M. Serum carotenoids and tocopherols and severity of nuclear and cortical opacities. *Investigative Ophthalmology & Visual Science* (1995), 36 (2), 276-288.

Marks, D.B., Marks, A.D., Smith, C.M. eds. *Basic Medical Biochemistry: A Clinical Approach* 1996.

Martinez, M.V., Whitaker, J.R. The biochemistry and control of enzymatic browning. *Trends in Food Science & Technology* (1995), 6 (6), 195-200.

Masarei, J.R.L., Puddey, I.B., Rouse, I.L., Lynch, W.J., Vandongen, R., Beilin, L.J. Effects of alcohol consumption on serum lipoprotein-lipid and apolipoprotein concentrations. *Atherosclerosis* (1986), 60 (1), 79-87.

Maxwell, S.R., Lip, G.Y.H. Free radicals and antioxidants in cardiovascular disease. *British Journal of Clinical Pharmacology* (1997), 44 (4), 307-317.

Maxwell, S., Thorpe, G. Tea flavonoids have little short term impact on serum antioxidant activity. *British Medical Journal* (1996), 313 (7051), 22.

Mazzetti, I., Grigolo, B., Borzi, R.M., Meliconi, R., Facchini, A. Serum copper/zinc superoxide dismutase levels in patients with rheumatoid arthritis. *International Journal of Clinical & Laboratory Research* (1996), 26 (4), 245-249.

McDowell, I.F., McEneny, J., Trimble, E.R. A rapid method for measurement of the susceptibility to oxidation of low-density lipoprotein. *Annals of Clinical Biochemistry* (1995), 32 (Pt 2), 167-174.

Miller, N.J., Castelluccio, C., Tijburg, L., Rice-Evans, C. The antioxidant properties of theaflavins and their gallate esters – radical scavengers or metal chelators? *FEBS Letter* (1996), 392 (1), 40-44.

Miller, N.J., Rice-Evans, C.A. Antioxidant activity of resveratrol in red wine. *Clinical Chemistry* (1995), 41 (12), 1789.

Miller, N.J., Rice-Evans, C.A. Spectrophotometric determination of antioxidant activity. *Redox Report* (1996), 2 (3), 161-171.

Miller, N.J., Rice-Evan, C.A., Davies, M.J., Gopinathan, V., Milner, A. A Novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science* (1993), 84 (4), 407-412.

Miura, S., Watanabe, J., Tomita, T., Sano, M., Tomita, I. The inhibitory effects of tea polyphenols (flavan-3-ol derivatives) on Cu^{2+} mediated oxidative modification of low density lipoprotein. *Biological and Pharmaceutical Bulletin* (1994), 17 (12), 1567-1572.

Mohr, D., Bowry, V.W., Stocker, R. Dietary supplementation with coenzyme Q10 results in increased levels of ubiquinol-10 within circulating lipoproteins and increased resistance of human low-density lipoprotein to the initiation of lipid peroxidation. *Biochimica et Biophysica Acta*. (1992), 1126 (3), 247-54.

Morand, C., Crespy, V., Manach, C., Besson, C., Demigne, C., Remesy, C. Plasma metabolites of quercetin and their antioxidant properties. *American Journal of Physiology* (1998), 275 (1 Pt 2), R212-R219.

Morrissey, P.A., O'Brien, N.M. Dietary antioxidants in health and disease. *International Dairy Journal* (1998), 8, 463-472.

Moslen, M.T. Reactive oxygen species in normal physiology, cell injury and phagocytosis. In: Armstrong, D. *Free Radicals in Diagnostic Medicine*. New York: Plenum Press, 1994, pp 17-27.

Muggli, R. Free radical tissue damage: the protective role of antioxidant nutrient. In: Corongiu, F. Banni, S. Dessi, M.A., Rice-Evans, C. eds. *Free Radicals and Antioxidants in Nutrition*. London: Richelieu, 1993, pp 189-204.

Mukhtar, H. Wang, Z.Y. Katiyar, S.K., Agarwal, R. Tea components: antimutagenic and anticarcinogenic effects. *Preventive Medicine* (1992), 21 (3), 351-360.

Mulholland, C.W., Strain, J.J. Serum total free radical trapping ability in acute myocardial infarction. *Clinical Biochemistry* (1991), 24 (5), 437-441.

Musonda, C.A., Chipman, J.K. Quercetin inhibits hydrogen peroxide (H_2O_2)-induced NF-kappaB DNA binding activity and DNA damage in HepG2 cells. *Carcinogenesis* (1998), 19 (9), 1583-1589.

Nagano, S., Kurokawa, M., Ebara, T., Naito, H., Sakamaki, R., Furukawa, S., Hirano, T., Maeda, M., Tsuji, A. Decrease of serum ascorbic acid concentrations in patients with diabetic macroangiopathy. *Journal of Nutritional Science and Vitaminology* (1996), 42 (1), 1-9.

- Namiki, M. Chemistry of Maillard reactions: recent studies on the browning reaction mechanism and the development of antioxidants and mutagens. *Advances in Food Research* (1988), 32, 115-184.
- Nanjo, F., Goto, K., Seto, R., Suzuki, M., Sakai, M., Hara, Y. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. *Free Radical Biology and Medicine* (1996), 21 (6), 895-902.
- Nath, N. Chari, S.N., Rathi, A.B. Superoxide dismutase in diabetic polymorphonuclear leukocytes. *Diabetes* (1984), 33 (6), 586-589.
- Neuzil, J., Stocker, R. Free and albumin-bound bilirubin are efficient co-antioxidants for α -tocopherol, inhibiting plasma and low density lipoprotein lipid peroxidation. *Journal of Biological Chemistry* (1994), 269 (24), 16712-16719.
- Niki, E. Antioxidant defenses in eukariotic cells: an overview. In: Poli, G., Albano, E., Dianzani, M.U. eds. *Free Radicals: From Basic Science to Medicine*. Switzerland: Birkhäuser Verlag Basel, 1993, 365-373.
- Niki, E., Yamamoto, Y., Kamiya, Y. Role of uric acid, cysteine, and glutathione as chain breaking antioxidants in aqueous phase. *Chemistry Letters* (1985), 1267-1270.
- Noda, Y., Mori, A., Packer, L. Gliclaide scavenges hydroxyl, superoxide and nitric oxide radicals: an ESR study. *Research Communications in Molecular Pathology & Pharmacology* (1997), 96 (2), 115-124.
- Noroozi, M., Angerson, W.J., Lean, M.E. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. *American Journal of Clinical Nutrition* (1998), 67 (6), 1210-1218.
- Odin, A.P. Vitamins as mutagens: advantages and some possible mechanisms of antimutagenic action. *Mutation Research* (1997), 386 (1), 39-67.
- Olive, P.L., Banáth, J.P., Durand, R.E. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. *Radiation Research* (1990), 122 (1), 69-72.
- Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J. Cullen, M.R., Glass, A., Keogh, J.P., Meyskens, F.L., Valanis, B., Williams, J.H., Barnhart, S., Hammar, S. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *New England Journal of Medicine* (1996), 334 (18), 1150-1155.
- Östling, O., Johanson, K.J. Microelectrophoretic study of radiation-induced DNA damage in individual mammalian cells. *Biochemical and Biophysical Research Communications* (1984), 123 (1), 291-298.
- Packer, L. Ultraviolet radiation (UVA, UVB) and skin antioxidants. In: Rice-Evans, C.A., Burdon, R.H. ed. *Free Radical Damage and Its Control*. Netherland: Elsevier Science B.V., 1994, pp 239-255.
- Packer, L., Witt, E.H., Tritschler, H.J. Antioxidants properties and clinical applications of alpha-lipoic acid and dihydrolipoic acid. In: Cadenas, E., Packer, L. eds. *Handbook of Antioxidants*. New York: Marcel Dekker, Inc. 1996, pp 545-591.
- Padh, H. Vitamin C: newer insights into its biochemical functions. *Nutrition Review* (1991), 49 (3), 65-70.

Paganga, G., Al-Hashim, H., Khodr, H., Scott, B.C., Aruoma, O.I., Hider, R.C., Halliwell, B., Rice-Evans, C.A. Mechanisms of antioxidant activities of quercetin and catechin. *Redox Report* (1996), 2 (6), 359-364.

Pallett, K.E., Young A.J. Carotenoids. In: Alscher, R.G., Hess, J.L. eds. *Antioxidants in Higher Plants*. Florida: CRC Press, 1993, 1-30.

Panayiotidis, M., Collins, A.R. *Ex vivo* assessment of lymphocyte antioxidant status using comet assay. *Free Radical Research* (1997), 27 (5), 533-537.

Paolisso, G., D'Amore, A., Giugliano, D., Cewriello, A. Varricchio, M., D'Onofrio, F. Pharmacologic doses of vitamin E improve insulin action in healthy subjects and non-insulin-dependent diabetics. *American Journal of Clinical Nutrition* (1993), 57 (5), 650-656.

Parks, D.A., Granger, D.N. Contributions of ischemia and reperfusion to mucosal lesion formation. *American Journal of Physiology* (1986), 250 (6 Pt 1), G749-G753.

Phelps, S., Harris, W.S. Garlic supplementation and lipoprotein oxidation susceptibility. *Lipids* (1993), 28 (5), 475-477.

Pietta, P., Simonetti, P., Gardana, C., Brusamolino, A., Morazzoni, P., Bombardelli, E. Catechin metabolites after intake of green tea infusions. *Biofactors* (1998a), 8 (1-2), 111-118.

Pietta, P., Simonetti, P., Gardana, C., Brusamolino, A., Morazzoni, P., Bombardelli, E. Relationship between rate and extent of catechin absorption and plasma antioxidant status. *Biochemistry & Molecular Biology International* (1998b), 46 (5), 895-903.

Prasad, K., Laxdal, V.A., Yu, M., Raney, B.L. Antioxidant activity of allicin, an active principle in garlic. *Molecular and Cellular Biochemistry* (1995), 148 (2), 183-189.

Puppo, A. Effect of flavonoids on hydroxyl radical formation by Fenton-type reactions; influence of the iron chelator. *Phytochemistry* (1992), 31 (1), 85-88.

el-Rahman, A., Hammouda, M.A., Fakeir, A. Flow cytometric evaluation of erythrocyte response to oxidant stress. *Cytometry* (1995), 20 (1), 19-22.

Ren, L.H., Vasil'ev, A.V., Orekhov, A.N. Tertov, V.V., Tutel'ian V.A. Anti-atherosclerotic properties of higher mushrooms (a clinico-experimental investigation). *Voprosy Pitaniia* (1989), 1, 16-19.

Reyold, J.E.F. ed. *Martindale, The Extra Pharmacopoeia*. 30th ed. London: The Pharmaceutical Press, 1993.

Rice-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M., Pridham, J.B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research* (1995), 22 (4), 375-383.

Rice-Evans, C.A., Miller, N.J., Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology & Medicine* (1996), 20 (7), 933-956.

Rice-Evans, C.A., Sampson, J., Bramley, P.M., Holloway, D.E. Why do we expect carotenoids to be antioxidants in vivo? *Free Radical Research* (1997), 26 (4), 381-398.

Rimm E.B., Giovannucci, E.L., Willett, W.C., Colditz, G.A., Ascherio, A., Rosner, B., Stampfer, M.J. Prospective study of alcohol consumption and risk of coronary disease in men. *Lancet* (1991), 338 (8765), 464-468.

Robak, J., Gryglewski, R.J. Flavonoids are scavengers of superoxide anions. *Biochemical Pharmacology* (1988), 37 (5), 837-841.

Roitt, I.M. *Essential Immunology*. 8th ed. Oxford; London: Blackwell Scientific Publications, 1994.

Rotruck J.T. Pope A.L., Ganther H.E., Swanson A.B., Hafeman D.G., Hoekstra W.G. Selenium: biochemical role as a component of glutathione peroxidase. *Science* (1973), 179 (73), 588-90.

RxList. <http://www.rxlist.com/cgi/generic/simva.htm>. 1997.

Sakagami, H., Datoh, K., Fukuchi, K., Gomi, K., Takeda, M. Effect of an iron-chelator on ascorbate-induced cytotoxicity. *Free Radical Biology & Medicine* (1997), 23 (2), 260-270.

Sano, M., Takahashi, Y., Yoshino, K., Shimoi, K., Nakamura, Y., Tomita, I., Oguni, I., Konomoto, H. Effect of tea (*Camellia sinensis* L.) on lipid peroxidation in rat liver and kidney: a comparison of green and black tea feeding. *Biological and Pharmaceutical Bulletin* (1995), 18 (7), 1006-1008.

Schoenberg, M.H., Birk, D., Beger, H.G. Oxidative stress in acute and chronic pancreatitis. *American Journal of Clinical Nutrition* (1995), 62, 1306S-1314S.

Scott, B.C., Butler, J., Halliwell, B., Aruoma, O.I. Evaluation of the antioxidant actions of ferulic acid and catechins. *Free Radical Research & Communication* (1993), 19 (4), 241-253.

Scott, G. *Antioxidants in Science, Technology, Medicine and Nutrition*. Chichester: Albion Publishing, 1997.

Serafini, M., Ghiselli, A., Ferro-Luzzi, A. *In vivo* antioxidant effect of green and black tea in man. *European Journal of Clinical Nutrition* (1996), 50 (1), 28-32.

Serafini, M., Maiani, G., Ferro-Luzzi, A. Alcohol-free red wine enhances plasma antioxidant capacity in humans. *Journal of Nutrition* (1998), 128 (6), 1003-1007.

Sharpe, P.C., McGrath, P.C., McClean, E., Young, I.S., Archbold, G.P.R. Effect of red wine consumption on lipoprotein (a) and other risk factor for atherosclerosis. *Quarterly Journal of Medicine* (1995), 88 (2), 101-108.

Sheehy, T., Morrissey, P.A. Tocopherols. In: Sadler, M., Cabellero, B., Strain, J.J. eds. *The Encyclopedia of Nutrition*. London: Academic Press, 1998, pp 1878-1884.

Shigenaga, M.K., Ames, B.N. Oxidants and mitogenesis as causes of mutation and cancer: the influence of diet. *Basic Life Sciences* (1993), 61, 419-36.

Shiraki, M., Hara, Y., Osawa, T., Kumon, H., Nakayama, T., Kawakishi, S. Antioxidative and antimutagenic effects of theaflavins from black tea. *Mutation Research* (1994), 323 (1-2), 29-34.

Sies, H. Medical applications of antioxidants: an update of current problems. In: Poli, G., Albano, E., Dianzani, M.U. eds. *Free Radicals: From Basic Science to Medicine*. Switzerland: Birkhäuser Verlag Basel, 1993, 419-423.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* (1988), 175 (1), 184-191.

Singh, R.B., Downing, D. Antioxidants and coronary artery disease. *Journal of Nutritional & Environmental Medicine* (1995), 5 (3), 219-224.

Singh, R.B., Niaz, M.A., Ghosh, M., Beegum, R., Bishnoi, I., Agarwal, P., Agarwal, A. Dietary intake and plasma levels of antioxidant vitamins in health and disease: a hospital-based case-control study. *Journal of Nutritional & Environmental Medicine* (1995), 5 (3), 235-242.

Singh, S., Melkani, G.C., Rani, C., Gaur, S.P.S., Agrawal, V., Agrawal, C.G. Oxidative stress and metabolic control in non-insulin dependent diabetes mellitus. *Indian Journal of Biochemistry & Biophysics* (1997), 34 (6), 512-517.

Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In: Packer, L., ed. *Methods in Enzymology* Vol. 299. Orlando: Academic Press, 1999, pp 152-178.

Soleas, G.J., Diamanadis, E.P., Goldberg, D.M. Wine as a biological fluid: history, production, and role in disease prevention. *Journal of Clinical Laboratory Analysis* (1997), 11 (5), 287-313.

Stahelin, H.B., Gey, K.F., Eicholzer, M., Luden, E., Brenesconi, F., Thurneysen, J., Brubacher, G. Plasma antioxidant vitamins and subsequent cancer mortality in the 12 year follow up prospective Basel study. *American Journal of Epidemiology* (1991), 133 (8), 766-775.

Stampfer, M.J., Rimm, E.B. Epidemiologic evidence for vitamin E in prevention of cardiovascular disease. *American Journal of Clinical Nutrition* (1995), 62 (suppl), 1365S-1369S.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., Witztum, J.L. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *New England Journal of Medicine* (1989), 320, (14), 915-924.

Steinmetz, K.A. Vegetables, fruit, and cancer prevention: a review. *Journal of the American Dietetic Association* (1996), 96 (10), 1027-1039.

Stensvold, I., Tverdal, A., Solvoll, K., Foss, O.P. Tea Consumption. Relationship to cholesterol, blood pressure, and coronary and total mortality. *Preventive Medicine* (1992), 21, 546-553.

Stich, H.F. Teas and tea components as inhibitors of carcinogen formation in model systems and man. *Preventive Medicine* (1992), 21 (3), 377-384.

Stocker, R., Bowry, V.W. Tocopherol-mediated peroxidation of lipoprotein lipids and its inhibition by co-antioxidants. In: Cadenas, E., Packer, L. eds. *Handbook of Antioxidants*. New York: Marcel Dekker, Inc. 1996, pp 27-41.

Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N., Ames, B.N. Bilirubin is an antioxidant of possible physiological importance. *Science* (1987), 235 (4792), 1403-4046.

Stocks, J., Gutteridge, J.M., Sharp, R.J., Dormandy, T.L. The inhibition of lipid autoxidation by human serum and its relation to serum proteins and alpha-tocopherol. *Clinical Science & Molecular Medicine* (1974), 47 (3), 223-33.

Strain, J.J., Benzie, I.F.F. Diet and antioxidant defence. In Sadler, M., Caballero, B. and Strain, J.J. eds. *The Encyclopedia of Nutrition*. London: Academic Press, 1998a. pp 95-105.

Strain, J.J., Benzie, I.F.F. Antioxidant nutrients. In: Sadler, M.J. Saltmarsh, M. eds. *Functional Foods; the Consumer, the Products and the Evidence*. Cambridge: The Royal Society of Chemistry, 1998b, pp 74-79.

Sweetman, S.F., Strain, J.J., McKelvey-Martin, V.J. Effect of antioxidant vitamin supplementation on DNA damage and repair in human lymphoblastoid cells. *Nutrition & Cancer* (1997), 27 (2), 122-130.

Taylor, D. Herbal medicine at a cross roads. *Environmental Health Perspectives* (1996), 104 (9), 924-928.

Teichert, J., Preiss, R. HPLC-methods for determination of lipoic acid and its reduced form in human plasma. *International Journal of Clinical Pharmacology, Therapy & Toxicology* (1992), 30 (11), 511-512.

Thesis, R.C., Benedict, S.R. The determination of phenols in the blood. *Journal of Biological Chemistry* (1924), 61, 67-71.

Thomas, M.J. Urate causes the human polymorphonuclear leukocyte to secrete superoxide. *Free Radical Biology & Medicine* (1992), 12 (1), 89-91.

Thomas, M.J. The role of free radicals and antioxidants: how do we know that they are working? *Critical Reviews in Food Science and Nutrition* (1995), 35 (1&2), 21-39.

Thomas, S.R., Neuzil, J., Stocker, R. Inhibition of LDL oxidation by ubiquinol-10. A protective mechanism for coenzyme Q in atherogenesis? *Molecular Aspects of Medicine* (1997), 18 suppl. S85-S103.

Thurnham, D. I., Davies, J., Crump, B. J., Situnayake, R.D., Davis, M. The use of different lipids to express serum tocopherol: lipid ratios for the measurement of vitamin E status. *Annals of Clinical Biochemistry* (1986), 23 (Pt 5), 514-520.

Tice, R.R., Strauss, G.H.S. The single cell gel electrophoresis/comet assay: a potential tool for detecting radiation-induced DNA damage in humans. *Stem Cell* (1995), 13 (Suppl 1), 207-214.

Torun, M., Yardim, S., Goneng, A., Sargin, H., Menevsw, A., Simsek, B. Serum β -carotene, vitamin E, vitamin C and malondialdehyde levels in several types of cancer. *Journal of Clinical Pharmacy and Therapeutics* (1995), 20 (5), 259-263.

Traber, M.G. Biokinetics of vitamin E. In: Cadenas, E., Packer, L. eds. *Handbook of Antioxidants*. New York: Marcel Dekker, Inc. 1996, pp 43-61.

Van Acker, S.A.B.E., van den Berg, D.J., Tromp, M.N.J.L., Griffioen, D.H., van Bennekom, W.P., van der Vijgh, W.J.F., Bast, A. Structural aspects of antioxidant activity of flavonoids. *Free Radical Biology & Medicine* (1996), 20 (3), 331-342.

van het Hof, K.H., Kivits, G.A.A., Westrate, J.A., Tijburg, L.B.M. Bioavailability of catechins from tea: effect of milk. *European Journal of Clinical Nutrition* (1998), 52 (5), 356-359.

Vecchia, C.L., Negri, E., Franceschi, S., D'Avanzo, B., Boyle, P. Tea consumption and cancer risk. *Nutrition and Cancer* (1992), 17 (1), 27-31.

Venditti, P., Piro, M.C., Artiaco, G., Di Meo, S. Effect of exercise on tissue anti-oxidant capacity and heart electrical properties in male and female rats. *European Journal of Applied Physiology & Occupational Physiology* (1996), 74 (4), 322-329.

Vieira, O., Laranjinha, J.A., Madeira, V., Almeida, L. Cholesteryl ester hydroperoxide formation in myoglobin-catalyzed low density lipoprotein oxidation: concerted antioxidant activity of caffeic and p-coumaric acids with ascorbate. *Biochemical Pharmacology* (1998), 55 (3), 333-340.

de Vries, J.H., Hollman, P.C., Meyboom, S., Buysman, M.N., Zock, P.L., van Staveren, W.A., Katan, M.B. Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake. *American Journal of Clinical Nutrition* (1998), 68 (1), 60-65.

Vinson, J., Hsu, C., Possanza, C., Drack, A., Pane, D., Davis, K., Klock, C., Graser, K., Wang, X. Lipid peroxidation and diabetic complications: effect of antioxidant vitamins C and E. In: Armstrong, D. *Free Radicals in Diagnostic Medicine*. New York: Plenum Press, 1994, pp 430-432.

Virtanen, S.M., Aro, A. Dietary factors in the aetiology of diabetes. *Annals of Medicine* (1994), 26 (6), 469-78.

Wang, H., Cao, G., Prior R.L. Total antioxidant capacity of fruits. *Journal of Agricultural Food Chemistry* (1996), 44 (3), 701-705.

Wang, J. F., Zhang, J.J., Chen, W.W. Study of action of *Ganoderma lucidum* on scavenging hydroxyl radicals from plasma. *Journal of Traditional Chinese Medicine* (1985), 5 (1), 55-60.

Washko, P.W., Wang, Y., Leivne, M. Ascorbic acid recycling in human neutrophils. *Journal of Biological Chemistry* (1993), 268 (21), 15331-15335.

Watts, G.F., Castelluccio, C., Rice-Evans, C., Taub, N.A., Baum, H., Quinn, P.J. Plasma coenzyme Q (Ubiquinone) concentrations in patients treated with simvastatin. *Journal of Clinical Pathology* (1993), 46 (11), 1055-1057.

Wayner, D.D., Burton, G.W., Ingold, K.U., Barclay, L.R., Locke, S.J. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. *Biochimica et Biophysica Acta* (1987), 924 (3), 408-419.

Weber, P., Bendich, A., Machlin, L.J. Vitamin E and human health: rationale for determining recommended intake levels. *Nutrition* (1997), 13 (5), 450-460.

Weisburger, J.H. Nutritional approach to cancer prevention with emphasis on vitamins, antioxidants, and carotenoids. *American Journal of Clinical Nutrition* (1991) 53 (1 Suppl), 226S-237S.

Weisbuger, J.H. Tea antioxidants and health. In: Cadenas, E., Packer, L. eds. *Handbook of Antioxidants*. New York: Marcel Dekker, Inc., 1996, pp 469-486.

de Whalley, C.V., Rankin, S.M., Hoult, J.R.S., Jessup, W., Leake, D.S. Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. *Biochemical Pharmacology* (1990), 39 (11), 1743-1750.

Whitehead, T.P., Robinson, D., Allaway, S., Syms, J., Hale, A. Effect of red wine ingestion on the antioxidant capacity of serum. *Clinical Chemistry* (1995), 41 (1), 32-35.

Winyard P.G., Morris C.J., Winrow V.R., Zadi M., Blake, D.R. Free radical pathways in the inflammatory response. In: Rice-Evans, C.A., Burdon, R.H. eds. *Free Radical Damage and Its Control*. Netherland: Elsevier, 1994, pp 361-383.

Wiseman, S.A., Balentine, D.A., Frei, B. Antioxidants in Tea. *Critical Review in Food Science and Nutrition* (1997), 37 (8), 705-718.

World Cancer Research Fund. *Nutrition and the Prevention of Cancer: a Global Perspective*. American Institute for Cancer Research Food, 1997.

Yamane, T., Hagiwara, N., Tateishi, M., Akachi, S., Kim, M., Okuzumi, J., Kitao, Y., Inagake, M., Kuwata, K., Takahashi, T. Inhibition of azoxymethane-induced colon carcinogenesis in rat by green tea polyphenol fraction. *Japanese Journal of Cancer Research* (1991), 82 (12), 1336-1339.

Yamashita, K., Iizuka, Y., Imai, T., Namiki, M. Sesame seed and its lignans produce marked enhancement of vitamin E activity in rats fed a low alpha-tocopherol diet. *Lipids* (1995), 30 (11), 1019-1028.

Yamashita, K., Nohara, Y., Katayama, K. Namiki, M. Sesame seed lignans and gamma-tocopherol act synergistically to produce vitamin E activity in rats. *Journal of Nutrition* (1992), 122 (12), 2440-2406.

Yang, C.S., Wang, Z.Y. Tea and cancer. *Journal of the National Cancer Institute* (1993), 85 (13), 1038-1049.

Yang, T.T.C., Koo, M.W.L. Hypocholesterolemic effects of Chinese tea. *Pharmacological Research* (1997), 35 (6), 505-512.

Young, J.F., Nielsen, S.E., Haraldsdottir, J., Daneshvar, B., Lauridsen, S.T., Knuthsen, P., Crozier, A., Sandstrom, B., Dragsted, L.O. Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. *American Journal of Clinical Nutrition* (1999), 69 (1), 87-94.

Yu, B.P. Cellular defenses against damage from reactive oxygen species. *Physiological Review* (1994), 74 (1), 139-162.

Yu, G.P., Hsieh, C.C., Wang, L.Y., Yu, S.Z., Li, X. L., Jin, T.H. Green-tea consumption and risk of stomach cancer: a population-based case-control study in Shanghai, China. *Cancer Causes and Control* (1995), 6 (6), 532-538.

Zhang, D., Yasuda, T. Yu, Y., Zheng, P., Kawabata, T., Ma, Y., Okada, S. Ginseng extract scavenges hydroxyl radical and protects unsaturated fatty acids from decomposition caused by iron-mediated lipid peroxidation. *Free Radical Biology & Medicine* (1996), 20 (1) 145-150.

Zhang, J., Shen, X. Antioxidant activities of baicalin, green tea polyphenols and alizarin *in vitro and in vivo*. *Journal Nutritional & Environmental Medicine* (1997), 7, 79-89.

Zhao, J., Li, X.J., He, R., Cheng, S., Wenjuan, X. Scavenging effect of extracts of green tea and natural antioxidants on active oxygen radicals. *Cell Biophysics* (1989), 14, 175-185.

Zhizhina, G.P., Blyukhterova, N.V. Effect of metal ions and xenobiotics on endogenous oxidation of DNA. *Biochemistry (Moscow)* (1997), 62 (1), 88-94.

Appendix 1

Ferric Reducing / Antioxidant Power Assay (FRAP) Protocol

Stock Reagents Preparation:

1. H_2O : either distilled or miliRO.
2. Acetate Buffer (300 mmol/l): 3.1 g sodium acetate trihydrate (Riedel-de Haen) in around 600 ml distilled water + 16 ml glacial acetic acid (BDH). Make up to 1 L with distilled water. pH should be 3.6.
3. FeCl_3 solution: 270 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (BDH) in 50 ml H_2O .
4. HCl solution (36 mmol/l): 1 ml conc HCl (Merck) in 250 ml H_2O .
5. 2,4,6-Tripyridyl-s-triazine (TPTZ) solution: 78 mg TPPZ (Fluka) in 25 ml 36mmol/l HCl
6. 1000 mmol/l FeSO_4 Standard: 27.8 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck) in 100 ml H_2O .

FRAP working reagent preparation:

20 ml acetate buffer + 2 ml FeCl_3 solution + 2 ml TPTZ solution; prepare as needed

FRAP FeCl_3 free working reagent preparation:

22 ml acetate buffer + 2 ml TPTZ solution; prepare as needed

Appendix 2**COBAS FARA Test Programme for FRAP & FRASC Assays**

Measurement mode: Abs

Reaction mode: R1-l-S-A

Reagent Black: reagent/dil

Wavelength: 593 nm

Temperature: 37.0 °C

R1: 300 µl

M1: 1.0 s

Sample Volume: 10 µl

Diluent Name: H₂O

Diluent Volume: 30 µl

First Reading: 0.5 s

Number of Reading: 25

Reading Interval: 15 s

Reaction Direction: increase

Calculation: endpoint

First Reading for Calculation: M1

Last Reading for Calculation: 17 (i.e. 4 min) for FRAP
5 (i.e. 1 min) for ascorbic acid

Appendix 3

Determination of Total Phenolic Compounds Protocol

1. Add 100 μl heparinised whole blood, urine sample or standard to 700 μl distilled water.
2. Mix, then add 100 μl 0.34 mol/l sodium tungstate (10% w/v).
3. Mix and allow to stand for 3 min.
4. Add 100 μl 0.33 mol/l H_2SO_4 and allow to stand for 10 min.
5. Centrifuge 3000 rpm for 10 min; remove 200 μl supernatant into clean test tube.
6. Add 800 μl distilled water to 200 μl supernatant.
7. Then add 100 μl Folin-Ciocalteu reagent (Sigma) and 200 μl 1.87 mol/l Na_2CO_3 (20% w/v).
8. Mix, allow to stand for 2 hours at room temperature.
9. Measure absorbance at 660 nm.
10. Read result from the standard curve prepared for working phenol standards.

Preparation of Stock Phenol Standards

Dissolve 1.506 g phenol (m.w. 94.11) in 1 L distilled water to give 16.0 mmol/l. Dilute stock solution in water to prepare working solution as required. Working standards concentration range from 2.3 mmol/l to 16.0 mmol/l.

Appendix 4

The Hong Kong Polytechnic University

Faculty of Health & Social Studies	
From:	Dr. Iris Benzie, Chairperson, FHSS Ad Hoc Human Ethics Subcommittee Room GH519; x 6394
To:	Professor Marion Edwards, OR
Re:	Application for human ethics approval for the project entitled: Effect of diet, disease and drugs on pro-oxidant:antioxidant balance <i>205</i>
I would be grateful if you would read through the attached application and let me have your comments/approval with regard to the following:	
Information given clear enough to ensure understanding by volunteers	<i>Revised</i> (yes) (no)
Risks and benefits clearly described	(yes) (no)
Risks minimal and/or outweighed by benefits	(yes) (no)
Confidentiality issue addressed adequately	(yes) (no)
Statement re Ethics Committee/complaint mechanism included	(yes) (no)
PI name and contact no. for further info given	(yes) (no)
Statement regarding 'withdrawal at any time with no penalty' included	(yes) (no)
Steps to gain informed consent adequate	(yes) (no)
I recommend that this application should be: approved <input type="checkbox"/> not approved <input type="checkbox"/> approved subject to minor revisions detailed on application <input checked="" type="checkbox"/>	
Other comments <i>Chinese translation of information in consent form will be needed in due course</i>	
Signed	Date <i>21.11.96</i>

21/11/96 I have seen the revised information sheet and it is now clear.

Dr Iris F.F. Benzie
11/20/96

Ethics approval should now be granted.

Dr. Marion Edwards

Hong Kong Polytechnic University
Faculty of Health and Social Studies

Ethics Review Checklist

Project Title: *Effect of Diet, Diet and Drugs on Probiotic Anticancer Balance*

Dept: *HS*

Principal Investigator/Chief Supervisor: *Dr. IRIS GENIE*

Tel: *6394*

Student Investigator:

Before completing this form, please refer to the guidelines on ethics review. As the Principal Investigator/Chief Supervisor, you are responsible for exercising appropriate professional judgment in this review.

- | | Yes | No |
|--|-------------------------------------|-------------------------------------|
| 1. Does the study involve subjects who are unable to give informed consent? (e.g. children, mentally handicapped people, unconscious patients) | <input type="checkbox"/> | <input checked="" type="checkbox"/> |
| 2. Are drugs or placebo to be administered to the subjects? | <input checked="" type="checkbox"/> | <input type="checkbox"/> |
| 3. Will blood or tissue samples be obtained from subjects? | <input checked="" type="checkbox"/> | <input type="checkbox"/> |
| 4. Is pain or more than mild discomfort likely to result from the experiment? | <input type="checkbox"/> | <input checked="" type="checkbox"/> |
| 5. Could the experiment/study induce undue (psychological) stress or anxiety? | <input type="checkbox"/> | <input checked="" type="checkbox"/> |
| 6. Will the study involve prolonged and repetitive testing? | <input checked="" type="checkbox"/> | <input type="checkbox"/> |
| 7. Will financial inducements (other than reasonable expenses & compensation for time) be offered to subjects? | <input type="checkbox"/> | <input checked="" type="checkbox"/> |
| 8. Will deception of subjects be necessary during the study? | <input type="checkbox"/> | <input checked="" type="checkbox"/> |
| 9. Will you be using ionising radiation? | <input type="checkbox"/> | <input checked="" type="checkbox"/> |

If you have checked "yes" to any of the above items, you must submit your proposal, together with an application form, to the Ethics Committee.

10. What data collection procedures will you be using? (Please list them here.)
some demographic data from volunteer; otherwise biochemical test results.

11. Will you be using a consent form? *YES*

If 'Yes' you must submit a copy of the consent form to the Ethics Committee.

If 'No' please state reasons:

ALL MATERIALS SUBMITTED WILL BE TREATED CONFIDENTIALLY.

NOTE: When any doubt arises in relation to the above, always forward an application to the Ethics Committee.

Signed:

Principal Investigator/Chief Supervisor
Student Investigator

Date: *19/1/01*

Research Study Information Sheet

Title of Project:

Antioxidant Properties of Tea and Chinese Herbal Medicines: a study of dietary strategies to improve antioxidant defence status and reduce oxidative stress.

Project Leader:

Dr IFF Benzie (Dept of Nursing & Health Sciences, GH519, Tel: 27666394)

Why is the study being performed?

The main purpose of the project is to assess the bioavailability of antioxidants in tea.

What do volunteers for the study have to do?

If you volunteer for the study you will be asked:

1. To sign an informed consent form that states you understand the information presented on this sheet. And give information about you (name, sex, age).
2. To give several blood and urine samples before and after ingestion of tea.
3. You are requested to fast overnight and blood and urine samples will be taken before the experiment. Then you will be given 2 cups of very strong tea (about 400 ml). After ingestion of tea, blood will be taken at 20, 40, 60 and 120 min. Urine sample is collected every 30 min up to 3 hours. You are requested to keep fasting before the last sample is collected but water is allowed.

Can a volunteer withdraw from the study?

Yes, you can stop participating in the study at any time with no penalty.

Can I get more information on the study?

Yes, contact Szeto Yim Tong Savio at 27667913 and he will try to answer any questions you may have.

* This study was approved by the Ethics Sub-Committee of the Hong Kong Polytechnic University. However, if you think there are any procedures that seem to violate your welfare, you may be complain in writing to the Ethics Sub-Committee of the Hong Kong Polytechnic University.

Consent Form

I agree to take part in the project entitled:

Antioxidant Properties of Tea and Chinese Herbal Medicines: a study of dietary strategies to improve antioxidant defence status and reduce oxidative stress.

Dr Iris Benzie (Project Leader):

I have read and understood the information presented to me.

I have had an opportunity to ask questions about the study, and these have been answered to my satisfaction.

I realise I may not benefit personally from taking part in the study.

I realise I can withdraw from the study at any time with no penalty.

I realise that the results of this study may be published, but that my own results will be kept confidential, and that I will not be identified personally in any published work.

Name : _____ **Signature :** _____

Witness : _____ **Signature :** _____

Date: _____

Appendix 5

Protocol for the Comet Assay

Slide preparation

Precoat clean glass microscope slides with agarose by dipping into a staining jar containing melted 1% standard agarose solution (in water); drain off excess agarose; wipe the back clear and dry in a warm oven; store at room temperature in slide box.

Reagents preparation

1 % standard agarose in water

Add 10 ml cold distilled water to 0.1 g standard agarose (Sigma).
Put the mixture in boiling water bath until agarose is completely melted.

1 % standard agarose in PBS

Add 10 ml cold PBS to 0.1 g standard agarose (Sigma).
Put the mixture in hot water bath until agarose is completely melted.

1 % low melting point agarose in PBS

Add 10 ml cold PBS to 0.1 g low melting point agarose (Sigma).
Put the mixture in hot water bath until agarose melt completely.
Put the melted agarose solution at 40°C water bath before use.

* Keep all the following solutions at 4°C in the dark unless otherwise specifies.

Lysis solution

2.5 mol/l (146.1 g/L) NaCl (BDH)
0.1 mol/l (37.22 g/L) Na₂EDTA (Sigma)
10 mmol/l (1.211 g/l) Tris (Sigma)
Adjust pH to 10 with either solid NaOH, or a concentrated NaOH solution (Riedel-de Haen).
Add 0.5 ml Triton X-100 (Sigma) per 50 ml immediately before use.

Electrophoresis solution

0.30 mol/l (12 g/l) NaOH (Riedel-de Haen)
1 mmol/l (0.372 g/l) Na₂EDTA(Sigma)

Neutralising buffer

0.4 mol/l (48.44 g/l) Tris (Sigma)
Adjust pH to 7.5 with conc. HCl (Merck).

PBS buffer

Add 1 PBS tablet to 200 ml distilled water, pH 7.4 (Sigma); final composition of this solution is 0.01 mmol/l, pH 7.4.

Hydrogen peroxide solution

Stock solution: 10 μ l 30% hydrogen peroxide solution (Sigma) in 10 ml PBS to give 10.0 mmol/l.

60 μ mol/l H_2O_2 working solution: 50 μ l stock solution in 8.35 ml PBS. Further diluted to 45 and 30 μ mol/l in PBS, prepared just before use.

Histopaque 1077 (Sigma)

Stored at 4°C, used as received.

RPMI 1640 and Fetal Bovine Serum Mixture

18 ml RPMI 1640 (GibcoBRL)

2 ml Fetal Bovine Serum (keep in freezer until use) (GibcoBRL)

Mix just before use.

Test agents preparation

* Keep all the following solutions at 4°C in dark unless otherwise specify.

Ascorbic acid solution

Stock solution: 10 mmol/l (1.76 g/l) Ascorbic acid (Merck) solution in PBS (freshly prepared)

Working solutions: Stock solution diluted to 50, 100, 200, 400, 800, 1600, 2000, 2500 and 3000 $\mu\text{mol/l}$ in PBS. These solutions should be made immediately before use.

α -tocopherol solution

Stock solution: 10 mmol/l (4.20 g/l) α -tocopherol (Merck) solution in absolute ethanol (freshly prepared)

Working solutions: Stock solution diluted to 50, 100, 200 $\mu\text{mol/l}$ in PBS.

Uric acid solution

Stock solution: 10 mmol/l (1.80 g/l) uric acid (Sigma) solution in PBS (freshly prepared)

Working solutions: Stock solution diluted to 100, 200, 400, 600, 800, 1000 and 1600 $\mu\text{mol/l}$ in PBS.

Other polyphenol solution (quercetin, caffeic acid, Catechin, epicatechin, catechin gallate, epigallocatechin, epigallocatechin gallate, resveratrol)

Stock solution: 1 mmol/l polyphenol (Sigma) solution in PBS

Working solutions: Further diluted to 25, 50 and 100 $\mu\text{mol/l}$ in PBS.

Tea infusions (green and black tea)

Stock infusion: 5 % (w/v), add 20 ml of boiling distilled water to 1 g tea leaves, let stand for 10 min and filter.

Working infusions: Stock infusion diluted to 0.25, 0.125 and 0.0625 % in PBS.

Lingzhi infusions

Stock infusion: 0.02 % (w/v), add 100 ml of 0.005 mol/l NaOH to 2 mg Lingzhi powder, let mix for 10 min on roller mixer, centrifuge for 3700 rpm for 10 min at room temperature.

Working infusions: Stock infusion diluted to 0.002, 0.001 and 0.0005 % in PBS.