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The Hong Kong Polytechnic University

Department of Health Technology and Informatics

Possible Mechanisms of Green Tea Effects on Health via Modulation of Redox Balance and DNA Damage & Repair *In Vitro* and *In Vivo*

By

Cyrus Kin-chun, HO

A thesis submitted in partial fulfilment of the requirement for the degree of Master of Philosophy

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Chief supervisor: Professor BENZIE IFF

Co-supervisors: Dr. CHOI SW & Dr. SIU MF Parco

Certificate Of Originality

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Abstract

It has been previously reported that regular intake of green tea is associated with lower basal oxidation-induced damage to DNA and increased resistance of lymphocytic DNA to oxidant challenge. However, *in vitro* pro-oxidant, genotoxic and cytotoxic effects of green tea polyphenols at high concentrations have been reported in the literature. This differential effect observed in the *in vitro* and *in vivo* studies may be mediated by H_2O_2, which in large amounts is genotoxic, but in small amounts could activate the redox-sensitive Antioxidant Response Element (ARE) via the Keap-1/Nrf2 redox switch, inducing cytoprotection. This study, consisting of both *in vitro* and *in vivo* parts, aimed to test this hypothesis.

For the *in vitro* part of the study, pooled lymphocytes from venous blood of 5 healthy volunteers were incubated for 30 min at 37^{o}C in 1) freshly prepared green tea (Pre-rain Loong-cheng tea) solutions (0.005%, 0.01%, 0.05%w/v in PBS, with PBS as control), 2) tea solutions with addition of catalase (CAT) and, 3) tea solutions with addition of catalase and superoxide dismutase (CAT+SOD). The H_2O_2 concentrations of 0.005%, 0.01% and 0.05% tea solutions after 30 min at 37^{o}C were, respectively, 3, 7 and 52μM. Genoprotective effect of green tea on lymphocytic DNA (assessed using the Fpg-assisted comet assay) at lower doses, i.e. 0.005% and 0.01%, was observed but a damaging effect was seen at higher dose of 0.05% tea. Addition of enzymes to remove H_2O_2 lowered the DNA damage induced by the 0.05% tea solution, but did not result in loss of genoprotection seen with the low doses. Gene expression of *HMOX1*, *NRF2*, *KEAP1*,

BACH1, and *hOGG1* were not found to be altered in any treatments compared to corresponding PBS control.

For the *in vivo* part, a controlled human intervention trial was conducted to investigate the acute (up to 2 hours post-ingestion) effects of a single dose of 1.5% pre-rain Loong-cheng green tea and effects on fasting samples of 7 days of twice daily 1% tea intake. Subjects (n=16) were randomised to take either tea (n=8) or water (n=8) first, and after a 4-week washout period they were crossed over to the other treatment. Samples were collected pre- and post both treatments. Results showed that single dose and regular intake of green tea were associated with relative decreases of ~30% ($p<0.05$) in lymphocytic DNA damage in samples collected at 60min and 120min after the single dose, and in fasting samples after 7 days of regular intake of green tea. Furthermore, after 7 days' supplementation with green tea, lymphocytic hOGG1 activity (measured using a variation of the comet assay; $p<0.001$), and HO-1 protein expression ($p<0.05$) were increased. However, no significant changes in total antioxidant capacity (as the FRAP value) in plasma or in gene expression of ARE-related factors (*HMOX1*, *NRF2*, *KEAP1*, *BACH1*, *NQO1*, *GSTα*, and *XRCC5)* were observed. A significant, inverse correlation was seen between plasma catechin concentrations and oxidation-induced DNA lesions, and a positive correlation between catechins and hOGG1 activity was seen.

This is the first study to investigate the effect of green tea on redox-controlled adaptive cytoprotection in a controlled human intervention trial. Genoprotection by green tea was confirmed in both the *in vitro* and *in vivo* parts of the study, and the H_2O_2-induced

genotoxic effects of high dose tea were also confirmed in the in vitro part. However, results did not support the initiation of the Nrf2/ARE signalling pathway by green tea as we saw no changes in gene expression. The *in vitro* genoprotection seen with low dose tea could be due to direct antioxidant protection by green tea polyphenols, or to H_2O_2-independent ARE-induction. The supplementation-related effects could also be due to direct antioxidant effects of the absorbed catechins, but could be due to post-translational effects on hOGG1, through which DNA repair activity is enhanced, and HO-1 and its cytoprotective effects.

List of Published and Presented Work from This Thesis

Conference Papers

- An abstract and poster *"Dose-related Differential Effects of Green Tea on Human DNA in vivo"* in International Conference of Oxidative Stress: Havana-Redox 2011 organised by the Pharmacy & Food Sciences College of Havana's University and the Cuban Society of Pharmacology – 2011, Havana (Cuba)

- An abstract and poster *"Green Tea, Genoprotection, and DNA Damage: are effects mediated by dose-dependent generation of hydrogen peroxide and redox switching?"* in Redox Biology and Micronutrients: From signalling to translation and back organised by the Society For Free Radical Research – Europe 2011, Istanbul (Turkey)

Peer-reviewed Papers

- Fung ST, Ho CK, Choi SW, Chung WY, Benzie IFF. Comparison of catechin profiles in human plasma and urine after single dosing and regular intake of green tea (*Camellia sinensis*). Br J Nutr. 2012;30:1-9.
- Ho CK, Choi SW, Siu PM, Benzie IFF. Genoprotection and genotoxicity of green tea (*Camellia sinesi*s): two sides of the same redox coin?. Redox Report (Submitted).
- Ho CK, Choi SW, Siu PM, Benzie IFF. Effects of a single dose and seven days' supplementation with green tea on redox balance, DNA damage and repair in healthy adults. Antioxidant Redox Signalling (To be submitted)

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List of Abbreviations

H_2O_2	Hydrogen peroxide
8-oxoGua	8-oxoguanine
APS	Ammonium persulphate
BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1
BO_2^-	Metaborate anions
BSA	Bovine serum albumin
CAT	Catalase
CD3ε	Cluster of differentiation 3-epsilon chain
CD8β	Cluster of differentiation 8-beta chain
cDNA	Complementary deoxyribonucleic acid
CHL	Cholesterol
CO_2	Carbon dioxide
Ct	Cycle number of threshold
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECL	Enhanced chemiluminescent
ELISA	Enzyme-linked immunosorbent assay
EGCG	Epigallocatechin-3-gallate
ECG	Epicatechin-3-gallate
EGC	Epigallocatechin
EC	Epicatechin
FBS	Foetal bovine serum
Fe(III)/TPTZ	Ferric tripyridyltriazine
$FeCl_2.7H_2O$	Iron(II) chloride-7-water
$FeCl_3.6H_2O$	Iron(III) chloride-6-water
Fpg	Formamidopyrimidine DNA glycosylase

FRAP	Ferric Reducing Antioxidant Power
GSTα	Glutathione S-transferase α
H_2SO_4	Sulphuric acid
H_3BO_3	Boric acid
HCl	Hydrogen Chloride
HDL	High-density lipoproteins
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HO-1	Haem oxygenase 1
hOGG1	Human 8-oxoguanine DNA glycosylase
HRP	Horseradish peroxidase
hsCRP	High-sensitivity C-reactive protein
KCl	Potassium chloride
KEAP1	Kelch-like ECH-associated protein 1
$KHCO_3$	Potassium hydrogencarbonate
KOH	Potassium hydroxide
LMP	Low melting point
LOQ	Limits of quantification
$MgCl_2$	Magnesium chloride
MMLV	Moloney Murine Leukaemia Virus
Na_2EDTA	Disodium ethylenediaminetetraacetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH4Cl	Ammonium chloride
$NH_4Fe(SO_4)2.6H_2O$	Ammonuium iron(III) sulphate-6-water
NQO1	NAD(P)H quinone oxidoreductase 1
NRF2	Nuclear factor erythroid 2-related factor 2
PBS	Phophate buffered saline
PCR	The polymerase chain reaction
PVDF	Polyvinylidene Fluoride

SOD	Superoxide dismutase
RBC	Red blood cells
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute-1640
RT-qPCR	The real-time reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TG	Triglycerides
TMB	Tetramethylbenzidin
TPTZ	Tripyridyl triazine
Tris	Trisaminomethane
Ultraviolet	UV
w/v	Weight/volume
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5

Chapter 1
Literature Review

Introduction

Oxidative stress, an imbalance between oxidants and antioxidants in favour of oxidants, has been implicated in the development of various diseases, including the common age-related diseases, cancer, cardiovascular disease, diabetes mellitus and Alzheimer's disease. Together, these bring a heavy socioeconomic burden to our society, and increasing prevalence of oxidative stress- associated diseases is a major issue, especially in our ageing population. Increased intake of dietary antioxidants is thought to help lower the risk of these oxidative stress- associated diseases, although the evidence for benefit is mainly through epidemiological studies. Antioxidants are substances that help delay or prevent the oxidation of an oxidisable substrate. Some antioxidants, such as antioxidant enzymes and glutathione, are synthesised endogenously, but others, notably vitamin C and vitamin E, must be supplied in the diet. There are many other dietary derived antioxidants, and polyphenols and carotenoids are the most numerous and common. There is as yet no known specific physiological role or deficiency state recognised for polyphenols or carotenoids, however, diets rich in these antioxidants are known to be beneficial to health.

Green tea (*Camellia sinensis*) is rich in polyphenolic antioxidants known as catechins, is a common component of the diet in Asia, and is an increasingly popular beverage worldwide. There are many reports from epidemiological studies that link green tea intake with lower risk of diseases associated with increased oxidative stress. The reported

benefits of green tea include promoting cardiovascular health, lowering lipids, and lowering cancer risk. However, well designed human experimental studies, especially studies looking at effects on DNA, are few and have shown conflicting results. Recent studies by our group showed a significant DNA protective effect by green tea both *in vitro* and in an '*in vivo'* (human supplementation) study. The purpose of the planned project was to build upon these interesting findings, and to take the investigation to a molecular level with the aim of studying a possible mechanism of action through a redox sensitive gene promoter region called the Antioxidant Response Element (ARE) (also known as the Electrophile Response Element, EpRE). This was studied through *in vitro* experiments and a controlled intervention trial with healthy adults.

Overview of Oxidative Stress:

Introduction:

The world's population has grown exponentially in the last half-century. In 1950, there were two and a half billion people, but currently there are over seven billion people living in the world (WHO, 2010). The fastest rates of population growth are in Africa and Asia, and the group of people whose number is increasing fastest is the elderly (WHO, 2010). The increase in population is due both to a higher life expectancy at birth because of low neonatal and childhood mortality, and to the increase in life expectancy of older adults (Lutz *et al.*, 2008). These changes have resulted from better health care provision, improved public health strategies, and from better education and nutrition (Lutz *et al.*, 2008). However, longer life expectancy has brought a heavy burden of age-related

diseases and disability (Kirkwood, 2008; Christensen *et al.*, 2009). At present, the majority (53%) of the world's older persons (aged 60 or over) lives in Asia (UN, 2010; WHO, 2010). The challenge of modern medicine is to promote quality of life and to enable the elderly to live independently and healthily for as long as possible (Christensen *et al.*, 2009).

The free radical theory of ageing, in which free radicals, produced during, for example, aerobic respiration cause cumulative oxidative damage resulting in ageing and death, was initially proposed by Denham Harman in 1956 (Harman, 1992). Since then the discovery of the enzyme superoxide dismutase (SOD) and the finding that oxidative stress increases with age have added some credibility to this theory (McCord & Fridovich, 1969; Beckman & Ames, 1998). However, the exact role of oxidative stress in ageing is still unclear, and many other theories of ageing exist (Kowald & Kirkwood, 1996; Partridge, 2001; Viña *et al.*, 2007; Gruber *et al.*, 2008). Still, oxidative stress is implicated as an underlying factor in all age related diseases (Halliwell & Gutteridge, 2007). Therefore, modulation of oxidative stress by better defence or repair of oxidation-induced damage could, at least in theory, promote healthy ageing.

Oxidative stress: concept of free radicals

The Earth's atmosphere contains 21% oxygen. Aerobic organisms cannot live without oxygen, as oxygen is the final acceptor of hydrogen and electrons released during fuel breakdown and release of energy harnessed as ATP. This energy is needed in cells for homeostasis and to synthesise essential compounds, such as polyunsaturated fatty acids,

proteins and steroids (Holbrook & Ikeyama, 2002; Blokhina *et al.*, 2003). Although oxygen is involved in life-essential catabolism, it is also deleterious (Benzie, 2000 & 2003). Reactive oxygen and nitrogen species are generated due to aerobic metabolism, endogenous enzymes such as NADPH oxidase (NOX enzymes), and other biochemical reactions (Ferguson *et al.*, 2006; Halliwell, 2006 & 2007; Valko *et al.*, 2007). Although it is recognised that both reactive oxygen and reactive nitrogen species occur *in vivo*, they are both referred to as reactive oxygen species (ROS) in this report because reactive nitrogen species, such as nitric oxide and peroxynitrite, also contain oxygen (Ferguson *et al.*, 2006). ROS are reactive chemical species due to the presence of one or more unpaired electrons, and have a tendency to oxidise other species/molecules by removing an electron to achieve a state of stability (Halliwell, 2006 & 2007; Valko *et al.*, 2007). When ROS react with a non-radical species, the ROS will be quenched and the non-radical species will be oxidised by losing an electron (D'Autréaux & Toledano, 2007; Halliwell & Gutteridge, 2007). This, in turn, will make the non-radical species into another ROS, which can start a chain reaction for further oxidation of other non-radicals until two radicals react and terminate the chain reaction (Halliwell & Gutteridge, 2007).

The generation of ROS is inevitable in aerobic organisms, but our metabolism has evolved to make use of ROS, and they play crucial roles as signalling molecules to regulate the action of kinases needed for phosphorylation and gene transcription, in regulating cell death mechanisms, and in killing bacteria in body defence (Halliwell, 2006; D'Autréaux & Toledano, 2007; Halliwell & Gutteridge, 2007; Benzie & Wachtel-Galor, 2010). However, excessive generation of ROS causes cellular damage, as they are

capable of initiating chain reactions, targeting major biomolecules, e.g. DNA, proteins, and lipids, and thereby causing oxidation-induced damage to cells and tissues (Holbrook & Ikeyama, 2002; Kothen & Nyska, 2002; Halliwell & Gutteridge, 2007). This imbalance in the body's oxidative status, or "oxidative stress", is used to describe a state in which pro-oxidants predominate (Figure 1.1). As defined by Sies *et al.* (2005), oxidative stress is an "imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage". The term describes a metabolic condition of cells, organs, or the entire organism characterised by an oxidative overload (Sies *et al.*, 2005). In short, oxidative stress occurs in biological systems, i.e. our body, when ROS are in excess. This can be due to excessive production of ROS or inadequate antioxidant defence (Benzie, 2000 & 2003; Sies *et al.*, 2005). Excessive production of endogenous ROS can be due to, for example, inflammation, or intensive exercise, but ROS can also be generated from detoxification of drugs, from ultraviolet radiation, pollution and cigarette smoke (Limón-Pacheco & Gonsebatt, 2009).

At the DNA level, small changes in redox tone (oxidant: antioxidant balance), affect gene expression (Palmer & Paulson, 1997; Rahman & MacNee, 2000), while oxidation-induced damage to DNA can cause strand breaks, mutation, carcinogenic changes, altered homeostasis, or apoptosis (Rahman & MacNee, 2000; Szeto & Benzie, 2002). With regard to the potential deleterious effects and the accumulation of oxidative DNA damage caused by ROS, oxidative stress is therefore thought to be responsible for various age-related chronic and degenerative diseases (Williamson *et al.*, 2009).

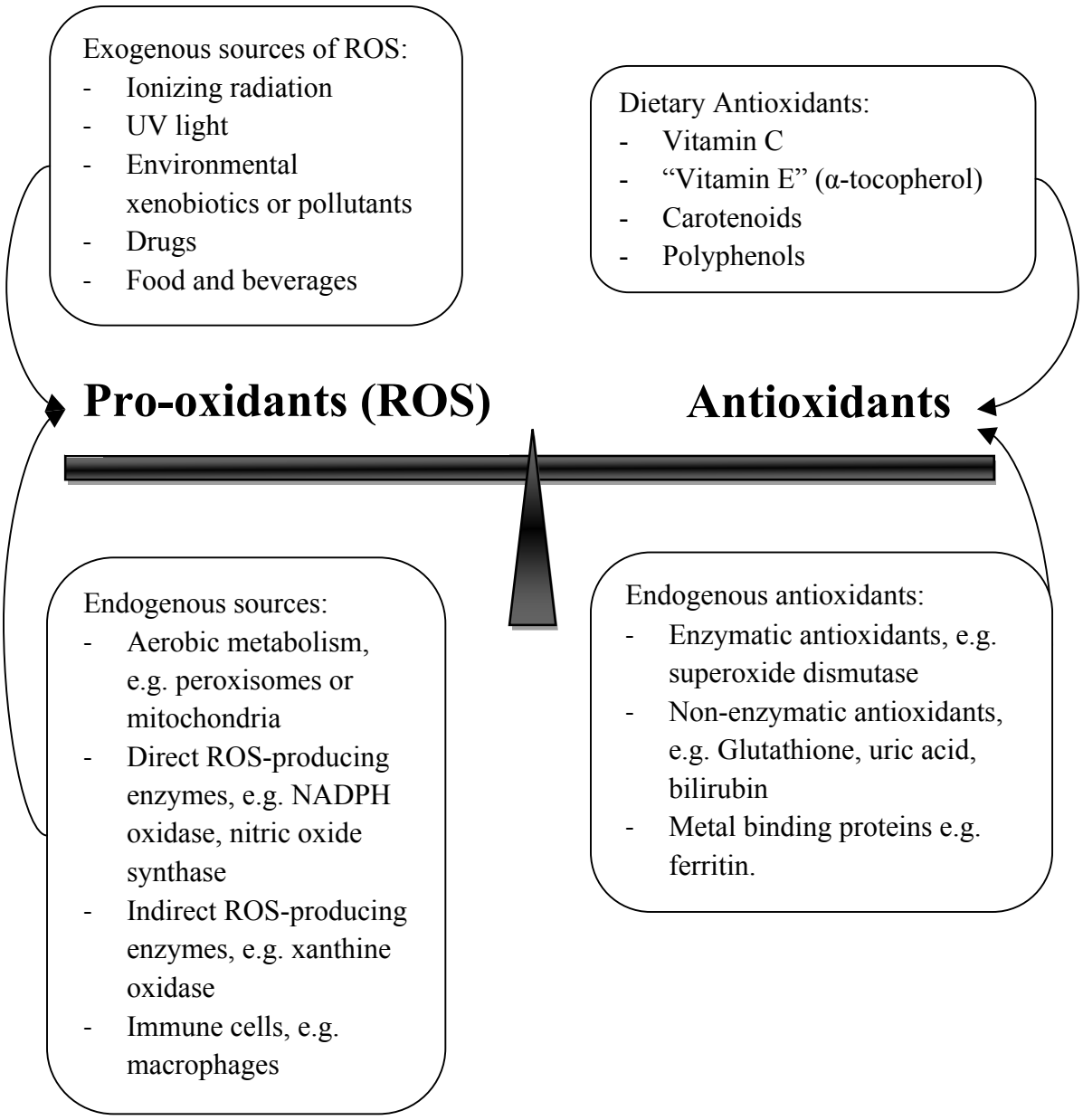


Figure 1.1 Oxidant: antioxidant balance

Oxidative stress and the antioxidant defence system

An antioxidant defence system has evolved in our bodies to deal with and lessen the deleterious effects of ROS (Benzie, 2000; Halliwell & Gutteridge, 2007). Various types of antioxidants are involved in the system. By definition, antioxidants are "substances that when present at low concentrations compared with those of an oxidizable substrate significantly delay or prevent oxidation of that substrate" (Halliwell & Gutteridge, 2007). Antioxidants are either synthesised endogenously or obtained from the diet. In general, antioxidants in our antioxidant defence system work as an integrated system. They help neutralise and eliminate the excessive ROS produced, thereby alleviating or avoiding oxidative-induced damage. Different strategies have evolved to eliminate excess ROS production, as follows (Benzie, 2000 & 2003):

1. Enzymatic removal of ROS by endogenous enzymes, such as catalase (CAT);
2. Non-enzymatic removal of ROS by direct scavenging (electron donation) antioxidants, such as glutathione (GSH) and ascorbic acid;
3. Avoiding pro-oxidant metals from approaching target biomolecules by the binding of these to proteins such as transferrin;
4. Quenching antioxidants such as carotenoids, which absorb energy.

Enzymatic antioxidants:

Superoxide Dismutases (SOD):

Superoxide is an ROS that is produced in the mitochondria during aerobic respiration and in the phagocytes when encountering foreign matter. The mitochondria are thought to be the major contributor of superoxide (>90%) in the body (Halliwell & Gutteridge, 2007).

The superoxide dismutases are the enzymes that catalyse the conversion of superoxide into hydrogen peroxide (Liochev & Fridovich, 2010). There are two types of SOD in human cells, CuZnSOD and MnSOD, where CuZnSOD is found mainly in the cytoplasm and nucleus, and MnSOD in mitochondria (McCord & Fridovich, 1969; Halliwell & Gutteridge, 2007). The reaction catalysed by both types is:

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

The hydrogen peroxide produced as a result of the above reaction is also an ROS. Therefore, another antioxidant, glutathione peroxidase, is responsible for the removal of the resulting hydrogen peroxide (Liochev & Fridovich, 2010).

Glutathione Peroxidase (GPx):

Glutathione peroxidase is a selenoprotein that is found in cytoplasm and mitochondria, and catalyses the oxidation of glutathione (GSH) (Brigelius-Flohé, 2006). Oxidation of GSH results from the coupling of hydrogen peroxide or other peroxides to give water molecules and glutathione disulphide (Brigelius-Flohé, 2006). Compared to superoxide, hydrogen peroxide is less reactive, but it is highly diffusible and can cause profound damage around the body because it can be broken down into hydroxyl radicals, another powerful type of ROS, giving rise to further oxidative damage (Brigelius-Flohé, 2006; Halliwell & Gutteridge, 2007). GPx catalyses this reaction;

$$H_2O_2 + 2GSH \xrightarrow{GPx} 2H_2O + GSSG$$

The oxidised glutathione, glutathione disulphide (GSSG), can be reduced back to GSH with the help of glutathione reductase (GR), another member of the antioxidant defence system, in the following reaction.

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

Catalase (CAT):

In addition to glutathione peroxidase, catalase is another enzyme that catalyses the breakdown of hydrogen peroxide to the less reactive oxygen and water (Cemeli *et al.*, 2009). CAT is mostly found in peroxisomes where high levels of H_2O_2 are found. High turnover rate of CAT allows its efficient catalytic action, protecting cells against oxidative damage (Cemeli *et al.*, 2009). The reaction (below) does not use GSH.

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Non-enzymatic antioxidants:

Glutathione (GSH):

Glutathione is a tripeptide, composed of glutamate, cysteine and glycine. In general, GSH concentration ranges from 0.4mM to 10mM in human tissues, but it is very low in plasma (Halliwell & Gutteridge, 2007). GSH results from the sequential catalysis by γ-glutamylcysteine synthetase and GSH synthetase (Halliwell & Gutteridge, 2007; Rahman & MacNee, 2000). GSH and its oxidised form, glutathione disulphide (GSSG), play a major role in regulating oxidant:antioxidant balance. As mentioned above, GSH can be oxidised to GSSG with the help of GPx in the presence of H_2O_2 or other peroxides. It has to be noted also that GSH itself can directly quench the actions of reactive species, e.g.

hydroxyl radicals and peroxyl radicals, by conjugation. On the other hand, GSSG can be oxidised back to GSH with the help of GR. The conversion between GSH and GSSG gives a ratio GSH/GSSG ratio, which is sometimes used as an indicator to determine the antioxidant:oxidant balance in the body (Unt *et al.*, 2008).

Uric acid:

Uric acid results from the breakdown of purines. It has been found to be a powerful scavenging antioxidant *in vitro* (Waring, 2002). Uric acid has been hypothesised to be able to protect cells against lipid peroxidation, although a high level has been associated with higher risk of insulin resistance and cardiovascular diseases (Waring, 2002; Halliwell & Gutteridge, 2007; Sautin *et al.*, 2007). An enzyme, uricase which cannot be synthesised in the human body, can lead to the oxidation of uric acid into allantoin. Allantoin found in plasma may be a biomarker of oxidative stress due to non-enzymatic oxidation of uric acid in reactions driven by free radicals (Kand'ár & Záková, 2008; Benzie & Strain, 1996; and Benzie *et al.*, 1999a).

Bilirubin:

Bilirubin is a lipid soluble product resulting from haem catabolism by haem oxygenase. Haem oxygenase, which accounts for 80% of bilirubin production, catabolises haem to biliverdin, and the endogenous biliverdin reductase further converts biliverdin to bilirubin. In general, daily production of bilirubin in a healthy adult is more than 270mg (Halliwell & Gutteridge, 2007). The antioxidant role of bilirubin was first proposed by Stocker and colleagues (1987). Though it is potentially cytotoxic, biliribin can help remove peroxyl

radicals in cells. Furthermore, bilirubin works in concert with α-tocopherol in protecting polyunsaturated fatty acids in membranes against oxidation-induced damage (Halliwell & Gutteridge, 2007).

Binding of proteins to pro-oxidant metals:

Transferrin:

Transferrin is an iron-binding glycoprotein. Chelation of iron can sequester free iron in the plasma and so avoid the oxidation reaction which is known as the Fenton reaction (Halliwell & Gutteridge, 2007). The Fenton reaction involves the production of hydroxyl radicals from peroxides where iron, in the form of Fe (II) ions, acts as a catalyst, as follows (Halliwell & Gutteridge, 2007).

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

Transferrin helps carry iron safely through the blood circulation. It releases iron into cells where it is taken up by another intracellular protein named ferritin, which also keeps the iron redox inactive (Balla *et al.*, 1992).

Ferritin:

Iron is taken up into cells by ferritin and stored in the form of Fe (II) ions, which will then be oxidised to Fe (III) with the release of hydrogen peroxide, as shown in the equations below (Balla *et al.*, 1992; Halliwell & Gutteridge, 2007). As endogenous antioxidants work in a network, the production of hydrogen peroxide can be removed by GPx or CAT (Halliwell & Gutteridge, 2007).

$$2Fe(II) + O_2 \rightarrow Fe(III) - O - O - Fe(III)$$

$$Fe(III) - O - O - Fe(III) \rightarrow Fe(III) - O - Fe(III) + H_2O_2$$

In summary, the human body has developed an antioxidant defence system which includes several different endogenous antioxidants to maintain a steady cellular redox balance to mitigate the deleterious effects of oxidative stress. However, excessive ROS generation can overwhelm our antioxidant defence, leading to an oxidant:antioxidant imbalance. It is not easy to upregulate the production of endogenous antioxidants, though this can occur due to cyto-adaptations to increased ROS. Increased intake of dietary antioxidant provides a more feasible approach to maintain or restore the oxidant:antioxidant balance in the face of oxidative stress.

Plant-based diets are rich in dietary antioxidants and there is evidence showing their effectiveness in combating degenerative and chronic diseases. These dietary antioxidants include vitamin C (ascorbic acid), 'vitamin E' (α-tocopherol), carotenoids and polyphenols. Among these, polyphenols, which are present in many plant-based food and beverages such as green tea, are suggested to play an important part in the protection against oxidative stress and age-related diseases.

Green Tea in Combating Oxidative Stress: the scientific evidence

Introduction:

Tea is one of the most widely consumed beverages in the world, due to its pleasant taste, aroma and appearance. It is especially popular in China and Japan (Mair & Hoh, 2009). Tea is made from the leaves of an evergreen shrub known as *Camellia sinensis*, a plant with dark-green leaves distributed in a mosaic manner along the stem and with fragrant white flowers, reaching to a height of up to 18 feet depending on the growing conditions (Weatherstone, 2007). *Camellia sinensis* is thought to have originated in South China and Southeast Asia as early as 5000 years ago, although more than 52 countries grow some variety of tea nowadays (Weatherstone, 2007). Tea has now become a popular, common beverage worldwide, and people are now more eager to drink tea due to its availability and the reported health benefits.

The manufacture of tea from *Camellia sinensis* is of economic and social importance. It gave rise to the trade between China and other parts of the world such as Europe and America, and the economic value of tea has been increasing in recent years due to the increase in tea production and consumption (Weatherstone, 2007). According to the Food and Agricultural Organization (FAO) of the United Nations (2010), the world tea production increased from 3.05 million tonnes in 2001 to 3.50 million tonnes by 2005, whereas the world tea consumption has also risen from 2.83 million tonnes in 2000 to 3.36 million tonnes by 2005. In addition to its economic importance, in the past tea was used for medicinal purposes, including relieving allergies, common colds and

gastroenteritis and is still believed to have many health promoting abilities (Jain *et al.*, 2006; Mair & Hoh, 2009; Serafini *et al.*, 2011b).

Green tea: tea production and processing

The three major varieties of teas are non-fermented tea (green tea), semi-fermented tea (oolong tea) and fermented tea (black tea) (Cabrera *et al.*, 2006). It is a common misunderstanding that green tea, oolong tea and black tea are made from different plants. These teas are actually all from the plant *Camellia sinensis*. The teas differ in the manufacturing processes of the leaves after picking, i.e. the degree of fermentation, which ultimately gives rise to the various flavours and appearances of the teas, and also leads to a marked difference in the polyphenolic content (Cabrera *et al.*, 2006; Huo *et al.*, 2008; Wang & Ho, 2009; Serafini *et al*., 2011b).

During the production of green tea, freshly picked tea leaves are initially steamed or pan-fried to inactivate polyphenol oxidase, and green tea leaves can then be collected after rolling and drying of heat-treated leaves (Crespy & Williamson, 2004). No oxidation of polyphenols will occur due to the lack of 'fermentation' of green tea. Therefore, the leaves retain their native green colour, and the most abundant amount of native polyphenolic compounds, the flavan-3-ols or catechins, is found in green tea among the three major varieties of teas (Huo *et al.*, 2008).

The production of black tea and oolong tea is different from that of green tea. It involves rolling (shaping) of leaves followed by fermentation (up to 6 hours), which will result in

the partial (for oolong tea) and more complete (for black tea) oxidation of the polyphenols found in the leaves by the action of polyphenol oxidase. This oxidation of polyphenols in leaves results in pigments (known as theaflavins and thearubigins), conferring the characteristic black colour of the tea. Fermentation is halted by further drying (reduction of water content) of leaves, ensuring a longer shelf-life. The production of oolong tea resembles a similar production process to that of black tea, but with a shorter fermentation process (partial oxidation), thus it is known as a 'semi-fermented' tea. Due to the semi-fermentation, it tastes less strong than black tea (Cabrera *et al.*, 2006).

Among the three different teas, black tea is usually preferred in western countries whereas green tea is favoured in Asian countries, such as China and Japan (Cabrera *et al.*, 2006; Alexophoulos *et al.*, 2007; Lambert *et al.*, 2007). Green tea (*Camellia sinensis*) is known to be rich in polyphenolic antioxidants especially, a family of flavan-3-ols called catechins. A schematic representation of tea processing is shown in Figure 1.2.

Green tea:

Fresh tea leaves → Frying (Steamed/ Pan-fried) → Rolling → Drying

Oolong tea:

Fresh tea leaves → Withered → Shaken → Partial fermentation → Panning → Rolling → Drying

Black tea:

Fresh tea leaves →Withered → Rolling → Complete fermentation → Drying

Figure 1.2 Schematic processing of tea

Green tea: chemical composition

Tea is a major source of dietary polyphenols. Polyphenols are a large group of diverse phytochemicals naturally occurring in plants. This large group is divided into two major classes: flavonoids and phenolic acids. According to Packer (1999) and Manach *et al.* (2004), approximately 1g of polyphenols is consumed daily, which is much higher than other antioxidants, e.g. 59-115mg for vitamin C and 5.2-6.0mg for vitamin E, although polyphenol consumption varies greatly between individuals (Szeto *et al.*, 2002). Diets high in polyphenols, especially flavonoids, have been associated with lower risk of degenerative and chronic diseases, including cardiovascular disease, inflammation, cancer and diabetes mellitus (Iso *et al.*, 2006; Kuriyama *et al.*, 2006; Williamson *et al.*, 2009). It is known that plant-based foods and beverages provide a rich source of polyphenols, and green tea is considered to be a major source of dietary polyphenolic antioxidants (Hanhineva *et al.*, 2010).

Compared to other teas from *Camellia sinensis*, green tea retains most of the native

polyphenols in the tea leaves, i.e. flavan-3-ols, because of the difference in the manufacturing processes, and these contribute around 30-42% of the dry mass of green tea leaves (Lambert *et al.*, 2007; Clement, 2009; Crozier *et al.*, 2009; Thielecke & Boschmann, 2009). Most of the native polyphenols (~75%) in black tea are transformed into compounds such as theaflavins and thearubigin which result from the oxidative polymerisation by phenolic oxidase during fermentation (Cabrera *et al.*, 2006). Apart from the polyphenolic content in tea leaves, other constituents of tea include vitamins, amino acids and caffeine. A summary of the composition of green and black tea leaves is shown in Table 1.

Table 1.1 Composition of green tea leaves (modified from Khan & Mukhtar, 2007; Hodgson & Croft, 2010)

Compounds	% of dry weight of tea	
	Black tea	Green tea
Catechins	10	30-42
Theaflavins	3-6	/
Thearubigens	12-18	/
Non-catechin flavonols, such as quercetin and myricetin	6-8	5-10
Other flavonoids such as anthocyanins and isoflavones	8-11	2-4
Caffeine	2-5	3-6
Carbohydrates	15	10-15
Minerals	10	6-8
Amino acids	13-15	4-6

Flavan-3-ols, also known as catechins, belong to the family of flavonoids, which are a large group of major plant pigments produced for protection against photochemical damage and thus are ubiquitously found in plants. Catechins are a major constituent of polyphenols found in green tea, contributing to 70-85% of the total polyphenols in the tea, and are thought to be responsible for the possible benefits brought about by tea consumption (Cabrera *et al.,* 2006; Moore *et al.*, 2009). Besides, catechins are water-soluble and are responsible for the astringent and bitter taste of green tea infusion. As

noted, during manufacture of green tea, most of the native catechins in tea leaves are preserved due to the inactivation of the phenolic oxidase by dry heating or steaming at the initial step. Black tea and oolong tea contain two thirds less catechins than green tea. A cup of green tea contains between 50mg to 200mg of polyphenols, depending on the manufacturing processes, growth conditions and the time of harvesting (Cabrera *et al.*, 2006; Huo *et al.*, 2008; Moore *et al.*, 2009). The four main catechins are presented in Figure 1.3.

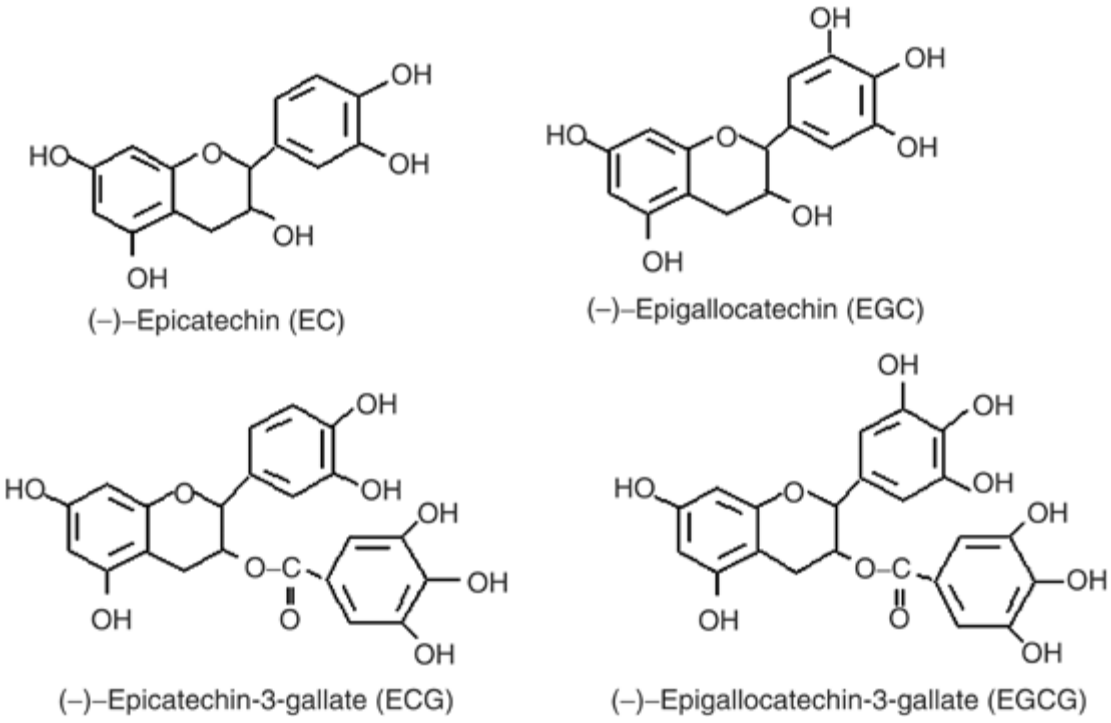


Figure 1.3 Chemical structures of the four major catechins identified in green tea: (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC) and epicatechin (EC) (Bigelow & Cardelli, 2006)

The signature of catechin structure is their dual benzene rings conjugated with a 3-carbon ring, in a configuration of C_6-C_3-C_6 (a common configuration of flavonoids). There are

four major catechins identified in green tea. They are (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), epicatechin (EC) and other epimeric forms of catechins. EGCG makes up approximately 60% of the total catechins content found in the tea (Cabrera *et al.*, 2006). The abundance of the four major constituents in green tea has been reported to be in the order of EGCG>EGC>ECG>EC (Cabrera *et al.*, 2006; Moore *et al.*, 2009). Human, animal and *in vitro* studies indicate that catechins contribute to the health benefits brought about by the consumption of green tea possibly due to the antioxidant power of the catechins (Cabrera *et al.*, 2006; Han *et al.*, 2011). The antioxidant effect of green tea is conferred by the presence of phenolic hydroxyl groups present on the catechins, and the number and arrangement of the hydroxyl groups determines the antioxidant power of the catechins due to its donation of hydrogen, stabilising excess free radicals in the body (Takeuchi *et al.*, 2007). It has been indicated that catechins in tea leaves can change upon heating and storage conditions. Tea catechins have been shown to degrade at a fast rate under high temperature, possibly due to oxidation and under an increased pH, especially EGCG and EGC which show complete degradation within six hours (Ito *et al*., 2003; Su *et al.*, 2003). Moreover, tea catechin content decreases to half of its original amount over 6-month storage in distilled water in the dark at room temperature, and the drop in content can be prevented by the presence of other additives such as citric acid and ascorbic acid (Su *et al.*, 2003).

Green tea: issues of bioavailability and biotransformation

High catechin content in green tea is thought to contribute to several potential health benefits, such as prevention of cancer and cardiovascular diseases (Cabrera *et al.*, 2006).

It is of importance to understand the bioavailability of catechins. The term 'bioavailability' has different definitions in different fields of interest, but in food and nutrition it can be defined as the rate and extent to which a compound is absorbed and becomes available to the site of action. In general, four major steps are involved: absorption, distribution, metabolism, and elimination.

Animal studies have shown that catechins can be absorbed and transferred to the target tissues (cells) for action (Moore *et al.*, 2009). Human studies have shown that consumption of green tea can elevate the total plasma antioxidant level within 40 minutes (Benzie *et al.*, 1999b). Catechins and the corresponding derived metabolites have been reported to reach their concentration peaks in plasma after 1.2-2.4 hours post green tea consumption, and catechin metabolites are detected within 4 hours in urine after consumption of a single dose of green tea in human studies (Henning *et al.*, 2004; Del Rio *et al.*, 2010b). Though it seems catechins are rapidly absorbed, studies have shown that the bioavailability of catechins is poor, as low as 4-10% (Stalmach *et al.*, 2009; Del Rio *et al.*, 2010a; Serafini *et al.*, 2011a). This reported low bioavailability might be caused by technical limitations, such as failure in recognising and identifying the metabolites originating from tea during analysis, failure in determining catechin metabolites which degrade rapidly, and to rapid metabolism of the catechins (Henning *et al.*, 2004).

From a human feeding study conducted by Del Rio's group (2010b), the bioavailability of catechins has been reported to be at least 40% with colonic ring fission catechin

metabolites taken into account. Catechins will undergo biotransformation and modification once ingested and absorbed. They are mainly metabolised in the liver by the action of phase II metabolising enzymes, such as methyltransferases (COMT), glucuronosyltransferases (GLUT) and sulphotransferases (SULT), giving rise to catechin metabolites conjugated with methyl, glucuronide or sulphide groups in plasma and urine (Wang *et al.*, 2008; Moore *et al.*, 2009). Catechins with more than one conjugated group have also been identified (known as mixed metabolites), while some catechins remain in their free form instead of being conjugated, e.g. EGCG mainly appears in the free form in plasma (77% is in free form), in contrast to the lower levels of free forms of EGC (31%) and EC (21%) in plasma one hour after green tea consumption (Lee *et al.*, 2002). It has also been demonstrated that no EGCG metabolites, in either free or conjugated forms, have been found in urine; and over 95% of the EGC and EC were in conjugated forms in urine (Lee *et al.*, 2002; Del Rio *et al.*, 2010b). Apart from biotransformation in the liver with the aid of various enzymes, unabsorbed catechins undergo microbial-induced metabolism in the colon, resulting in the formation of ring fission metabolites including valerolactones. Del Rio and colleagues (2010b) have identified a total of 24 catechin metabolites (both colonic and non-colonic) by HPLC-MS/MS (summarised in Table 2), in a human feeding study in which a single drink of 400ml ready-to-drink green tea was ingested. Of the 24 metabolites found in urine, 8 were found in plasma. The group has also shown that non-colonic metabolites can be detected in urine within 4 hours post ingestion of green tea, whereas ring fission metabolites (colonic metabolites) are usually observed and identified only after at least 7 hours post ingestion. This is in agreement

with a previous human feeding study conducted by Lee's group (2002). A simplified diagram on the pharmacokinetics of catechins is shown in Figure 1.4.

After undergoing biotransformation, catechin metabolites may have different physiochemical properties compared to their parental forms in terms of their biological activity such as radical-scavenging and metal chelating ability, which are believed to be crucial to the reported health benefits of green tea. In most studies, animals have been used as a model for the investigation of the biological activities of the metabolites, and the cell culture model is another commonly adopted experimentation approach. Generally, it is thought that biotransformation may lead to a decrease in biological activity (Moore *et al.*, 2009). However, biotransformed compounds have also been found to have a similar biological activity compared with the parental ones in certain cases. As reported by Lu *et al*. (2003) using the colon cancer cell line HT-29, EGC-3'-glucuronide has a lower radical scavenging activity than its parent compound EGC measured by 1,1-diphenyl-2-picrylhydryl (DPPH) radical assay, whereas EGCG-3'-glucuronide has a similar activity compared to its parent compound EGCG.

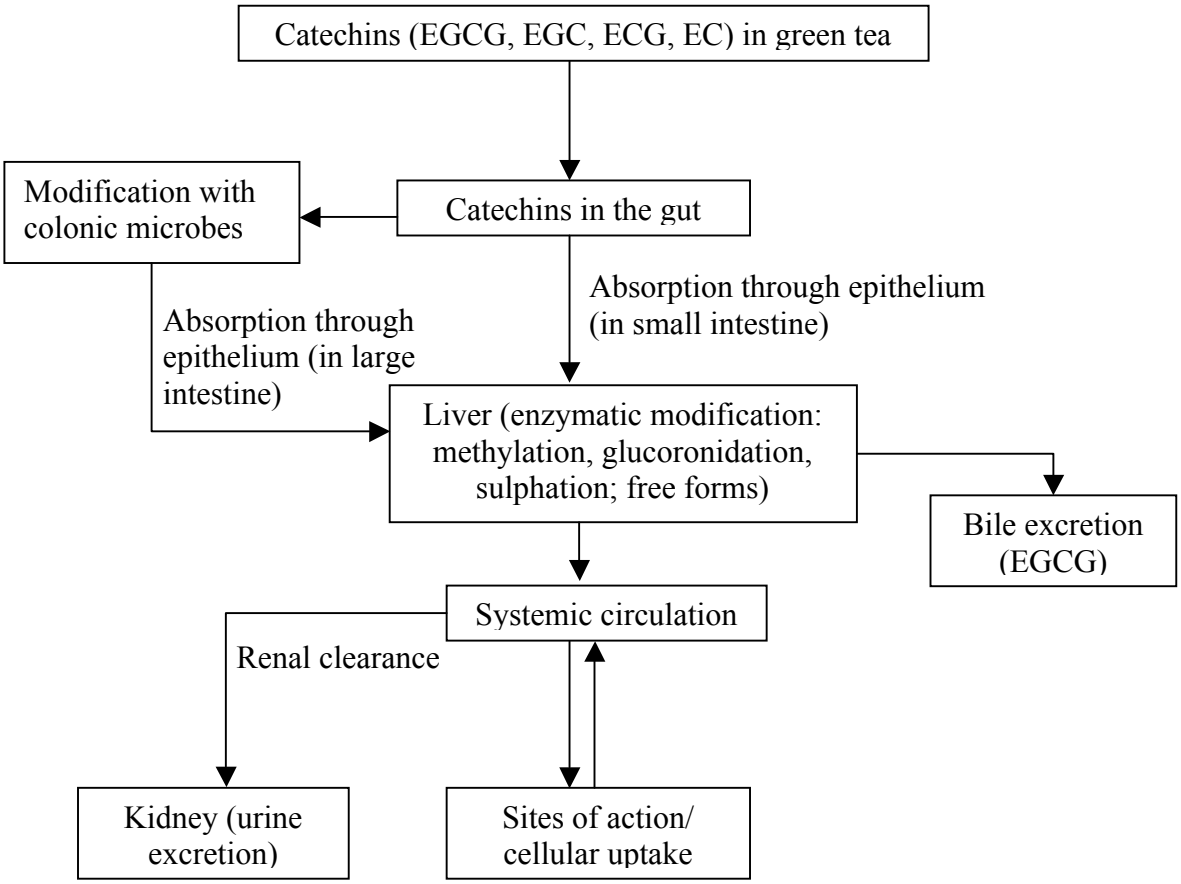


Figure 1.4 Simple representation of administration, distribution, metabolism, and elimination of catechins

Table 1.2 Catechin metabolite profile identified in plasma (a), and in urine (b), after single administration of 400ml of a ready-to-drink green tea by HPLC-MS/MS, where M represents valerolactones (from Del Rio *et al.*, 2010b)

(a) Compounds	**Peak plasma concentrations (nmol/l)**
(-)-Epigallocatechin-3-gallate	79.9
(-)-Epicatechin-3-gallate	Data not shown
(Epi)catechin-glucuronide	29.2
(Epi)catechin-sulphate	Data not shown
(Epi)gallocatechin-glucuronide	40.5
Methyl-(epi)catechin-sulphate	14.2
Methyl-(epi)gallocatechin-glucuronide	24.6
Methyl-(epi)gallocatechin-sulphate	38.0

(b) Compounds	**Metabolite excreted (µmol)**
(Epi)catechin-glucuronide	1.29
(Epi)catechin-sulphate	1.44
(Epi)catechin-sulphate glucuronide	0.20
(Epi)gallocatechin-glucuronide	4.39
(Epi)gallocatechin-sulphate	0.08
(Epi)gallocatechin-sulphate glucuronide	Data not shown
M4-glucuronide	4.81
M4-sulphate	9.68
M6-/M6'-disulphate	0.01
M6-/M6'-glucuronide	19.52
M6-/M6'-sulphate	91.21
M6-/M6'-sulphate glucuronide	1.07
Methyl-M4-sulphate	19.05
Methyl-(epi)catechin-glucuronide	Data not shown
Methyl-(epi)catechin-sulphate	1.99
Methyl-(epi)catechin-sulphate glucuronide	Data not shown
Methyl-(epi)gallocatechin-glucuronide	2.93
Methyl-(epi)gallocatechin-sulphate	1.63
Methyl-(epi)gallocatechin-sulphate glucuronide	0.37

Green tea consumption and health promotion: evidence from epidemiological and experimental (human intervention) studies

Green tea consumption has been suggested to be involved in the prevention and alleviation of degenerative and chronic diseases, such as diabetes mellitus, cancer, cardiovascular diseases, and obesity (Jain *et al.*, 2006; Williamson *et al.*, 2009). It has also been suggested that its particular involvement in combating diseases may be associated with ameliorating oxidative stress by improving the plasma antioxidant status. However, this is likely to be too simple a view (Benzie & Wachtel-Galor, 2010) Catechins are the main bioactive components in green tea, and the reported health benefits are attributed to the high catechin content (or possibly their metabolites), especially the most abundant catechin, epigallocatechin-3-gallate (EGCG). Research on the relationship among green tea consumption, antioxidants and health is intense and vigorous, including human, animal and cell culture studies. However, no definite conclusion on beneficial effects of green tea on health promotion can be drawn due to the high variations among subjects and the inconsistencies of the conducted methods and presented results. Here, possible health benefits in relation to green tea consumption will be discussed briefly with regard to different findings from epidemiological and experimental studies.

Cardiovascular protection:

Cardiovascular diseases (CVD) are a group of disorders related to heart and blood vessels, including coronary heart disease, cerebrovascular diseases and peripheral arterial disease. In 2004, approximately 17.1 million people worldwide died of cardiovascular diseases, contributing to 29% of all deaths (WHO, 2010). In Hong Kong, CVD accounted for 25%

of all mortality in 2008 (Department of Health, 2012). Atherosclerosis is one of the major pathophysiological factors for the development of CVD, and is related to hypertension, hypercholesterolemia and inflammation. This chronic inflammatory process involves the interactions among the white blood cells (monocytes), chemokines and endothelial cells of artery walls, eventually giving rise to atherosclerotic plaque formation. The oxidation of low-density lipoproteins (LDL), forming oxidised LDL (LDLox), by endogenous ROS has been associated with the initiation and progression of atherosclerosis with its ability to form lipid-laden foam cells and further leading to atherosclerotic plaques (Steinberg, 2005). Habitual green tea consumption has been shown to lower the risk of CVD (Kuriyama, 2008). This may be due to the high catechin content in green tea, maintaining a balanced lipid metabolism, restoring endothelial functions and protecting against ROS attack and thereby preventing the formation of atherosclerotic plaques (Hodgson & Croft, 2010).

Epidemiological studies conducted mainly in Asian countries have suggested a possible inverse association between green tea consumption and the risk of CVD (Nakachi *et al.*, 2000; Kuriyama *et al.*, 2006; Wang *et al.*, 2011). In a Japanese prospective cohort study, Kuriyama and colleagues (2006) reported that green tea consumption is inversely associated with decreased mortality resulting from CVD after investigating all-cause and cause-specific mortality. This inverse association of green tea consumption and decreased CVD mortality has been found to be more pronounced in women (Kuriyama *et al.*, 2006). The risk of CVD mortality was significantly decreased, by 31% in women and by 22% in men, in those drinking more than 5 cups of 100ml green tea a day (For women: Hazard

ratio (HR) = 0.69; 95% CI: 0.53-0.90; P-trend = 0.004; For men: HR = 0.78; 95% CI: 0.61-1.00; P-trend = 0.05) compared to those drinking one cup or no green tea a day (HR=1 as referent; Kuriyama *et al.*, 2006).

Human intervention studies have given further support to the cardiovascular protective effects of green tea consumption. In a randomised, double-blind, placebo-controlled human study conducted among 111 healthy subjects taking green tea extract containing 200mg decaffeinated catechins for 42 days, significant decreases in systolic blood pressure, total LDL and cholesterol concentrations were demonstrated (Nantz *et al.*, 2009). Another group has also shown a significant decrease in the level of oxidised LDL in plasma after a 4-week supplementation period with commercially available green tea among 20 male smokers (Lee *et al.*, 2005). This is supported by a human supplementation study conducted by Tinahones *et al.* (2008). The group has shown that 14 apparently healthy females had significant decreases in triglycerides and oxidised LDL levels after taking green tea extract containing 375mg catechins for 5 weeks (Tinahones *et al.*, 2008).

Cancer protection:

Cancer, caused by the accumulated mutations of cellular DNA and characterised by the uncontrolled growth and metastasis of abnormal cells, is a major cause of death globally. Approximately 7.9 million people worldwide died of cancer in 2007, accounting for 13% of all deaths (WHO, 2010). In particular in Hong Kong, cancer accounted for 31.2% of all deaths in 2007, which is much higher than the global percentage (Hong Kong Cancer Registry, 2007). Various factors contribute to the development of cancer including

environmental carcinogens, age, gender, and genetic factors (American Cancer Society, 2010). Conventionally, cancer is treated with three major therapies, surgery, radiotherapy, and systemic therapy (e.g. hormonal therapy, immunotherapy and chemotherapy). Apart from these therapies, dietary and supplementary approaches to treat cancers and even to prevent cancer have received much attention in the last decade, and the relationship between cancer incidence and green tea consumption has been investigated epidemiologically and experimentally.

Most of the epidemiological studies investigating the association between green tea consumption and cancer were conducted in Asian countries, such as Japan. To date, no concrete conclusion on green tea effects against cancer can be made. Imai *et al.* (1997) reported that daily consumption of 10 cups or more of green tea (an average of around 150ml per cup, with 10 cups providing approximately 300-400mg of EGCG per day), could delay the occurrence of breast cancer in a cohort study of over 8000 individuals. Nakachi *et al.* (1998) further showed that daily consumption of 5 cups or more of green tea could significantly lower the recurrence of breast cancer and prolong the cancer-free period in patients of stage I/II breast cancer. Another cohort study of 8552 people conducted by the same group and followed up for 9 years, showed that a lower risk of cancer incidence is associated with green tea consumption, especially in Japanese women who consume over 10 cups of green tea (~150ml/cup) daily (Nakachi *et al*., 2000). However, in another cohort study of 38540 people, no association between cancer incidence and green tea consumption was observed (Nagano *et al.*, 2001). This is supported by another cohort study conducted by Kuriyama *et al.* in 2006 which included

40530 people followed up for 7 years. The group showed no significant inverse association between green tea consumption and cancer incidence (Kuriyama *et al.*, 2006). Therefore, inconclusive results were seen from epidemiological studies. However, human intervention studies have provided crucial support for the protective effect of green tea consumption against cancers because of data indicating genoprotective effects (Boehm *et al.*, 2009).

In a randomised, double blind, placebo-controlled study with 124 adults with a high risk of suffering from liver cancer, a significant decrease in the urinary 8-hydroxydeoxyguanosine (8-oxodG; an oxidative stress marker, and a risk factor for cancer) was observed in the groups consuming either 500mg green tea polyphenol extract (GTP) or 1000mg GTP four times a day for 3 months (Wu *et al.*, 2004; Luo *et al.*, 2006). In another human intervention study that focusing on the protective effect of green tea consumption on skin cancer, Morley *et al.* (2005) reported that DNA damage induced by ultraviolet A (UVA) irradiation was found to be significantly lowered after consumption of three cups of 180ml 1.3% green tea infusion. Hakim *et al.* (2003) performed a randomised, placebo-controlled study focused on effects of tea consumption on oxidative DNA damage among 133 smokers (more than 10 cigarettes a day and having smoked for more than a year). The group demonstrated that 8-oxodG was significantly decreased in the green tea group after 4 months of consumption (4 cups a day; 235ml/cup) compared to water (placebo) group.

Diabetes mellitus:

Diabetes mellitus (DM) is a group of diseases characterised by high glucose levels resulting from defects in the body's ability to produce or use insulin (American Diabetes Association, 2010). It can be caused by an inability to produce insulin by the pancreas (type 1 diabetes) or the resistance of body cells to insulin, which means cells do not respond to insulin (type 2 DM) (American Diabetes Association, 2010). Type 2 DM is the most common form of DM, comprising 90% of the total DM patients, and progression of the disease may lead to severe complications such as renal failure (WHO, 2007). Oxidative stress has been proposed to be involved in the initiation and progression of the disease (Valko *et al.*, 2007). Accumulating evidence, in epidemiological studies, intervention studies and animal studies, shows that tea might help in diabetes, but the results from different groups or from different regions vary, and were not conclusive.

Epidemiological studies have been conducted to evaluate the association between green tea consumption and the risk of type 2 DM, but results were conflicting. In a Japanese cohort study with a total of 17413 volunteers (6727 men and 10686 women) aged 40-65 and not diagnosed with type 2 DM at baseline and followed-up for 5 years, Iso *et al.* (2006) reported a daily green tea consumption of six cups or more (200ml per cup) was accompanied with a lower risk of type 2 DM, of which the multivariable odds ratio was found to be 0.67 with 95% CI from 0.47-0.94. This inverse association was not seen with black tea or oolong tea consumption, and was more pronounced in women compared to men (Iso *et al.*, 2006). These results are partly in line with another study performed on elderly (aged>65 years) from the Mediterranean Islands (Panagiotakos *et al.*, 2009). The group showed that moderate daily consumption of green tea (1-2 cups; 150ml/cup) was

associated with a lower risk of suffering from type 2 DM with a multivariable odds ratio of 0.7 (95% CI: 0.41-0.86) (Panagiotakos *et al.*, 2009). Apart from an inverse association with type 2 DM, it was demonstrated that significant decrease in the fasting blood glucose concentration was observed in volunteers with moderate consumption of green tea ($p<0.01$ compared to non tea-drinkers) (Panagiotakos *et al.*, 2009). However, no association between tea consumption and the risk of type 2 DM has also been reported (Oba *et al.*, 2010). Oba and colleagues (2010) conducted a community-based cohort study with a total of 13540 participants (5897 men and 7643 women) aged younger than 70 years of age and with no diagnosed diabetes, cancer or CVD at baseline (in 1992). The group found that neither green tea nor black tea consumption was significantly associated with the risk of developing type 2 DM (Oba *et al.*, 2010).

Compared to conflicting results from epidemiological studies, human intervention studies have shown relatively clear supportive evidence of green tea consumption on glucose metabolism. Nagao *et al.* (2009) examined the effects of 12-weeks' supplementation with a catechin-rich beverage on glucose metabolism in a total of 43 type 2 DM patients in a randomised double-blinded controlled parallel study. Subjects were asked to take the beverages daily for 12 weeks after a 4-week run-in period, and were followed up for 4 weeks. Subjects in the catechin-rich group were required to drink the 340ml testing beverage prepared with green tea extract (with 582.8mg catechins) daily, whereas a control drink with 96.3mg catechin was given to those in the control group. A significant increase in insulin concentration was observed in the catechin-rich group when compared to both control group and baseline, but the decrease in glucose concentration and HbA1c

(glycated haemoglobin, a biomarker of glycaemic control) level between catechin and control groups did not reach significance. The group further examined patients prescribed with insulinotropic agents (either oral sulphonylureas or glinides) out of 43 subjects, with 16 in the catechin-rich group and 17 in control group. Insulin concentration in the catechin-rich group at week 12 was shown to be significantly higher than at baseline, and HbA1c level was significantly lowered in the catechin-rich group. The finding is consistent with a previous randomised, controlled human supplementation trial of crossover design of a total of 60 Japanese subjects (borderline diabetes with a fasting blood glucose level of >6.1mmol/l or a non-fasting blood glucose level of >7.8mmol/L) conducted in Japan by Fukino and colleagues (2008). The group showed a significant decrease in $HbA1_C$ after two-month's supplementation with green tea extract containing 544mg polyphenols. This may imply longer term green tea supplementation may help alleviate the medical conditions of individuals with less severe diabetes.

Obesity:

Obesity is a state of excess body fat accumulation, which can be defined by body mass index (BMI) over $25kg/m^2$ for Asians according to WHO (2010), and this affects an estimated 21% of the Hong Kong population (Department of Health, 2012). It is particularly prevalent in developed countries, and is considered to be a major risk factor for the development of many chronic diseases, including cardiovascular disease and DM. The effects of tea on alleviation of obesity has received much attention, and epidemiological studies have been carried out to investigate the association of tea consumption with body weight and markers related to obesity, such as BMI and waist-hip

ratio. Wu *et al.* (2003) reported that habitual tea consumption (at least once a week for over 6 months) may contribute to the decrease in percentage body fat after studying a cohort of 1103 Taiwanese. The group studied adults drinking tea for more than 10 years and surveyed their tea habits using structured questionnaires. In the study, approximately 96% of habitual tea drinkers mostly consumed oolong tea and green tea. Habitual tea drinkers were reported to have a lower percentage body fat (19.6% less) and waist-hip ratio (2.1% less) compared to non-habitual tea drinkers. In a report from the Netherlands Cohort Study with 4280 individuals aged 55-69 at baseline (2107 males and 2173 females) (Hughes *et al.*, 2008), a reverse association between flavonoid intake and BMI in women was shown. Subjects were divided according to gender into two groups and further split into five quintiles based on their reported flavonoid intake. It was observed that women with a higher intake of total flavonols/flavones and total catechins demonstrated a significantly ($p<0.05$) lower increase in BMI compared to those with lower intake, showing that flavonoid intake may help maintain the body weight in women whose BMI were in an increasing trend within the 14-year study period. No effects were seen in men with either a high or low intake of flavonoids in this 14-year study.

Anti-inflammatory effects:

Inflammation is thought to be related to the initiation and progression of atherosclerosis (Khan & Mukhtar, 2007; Moore *et al.*, 2009). Various factors are found to mediate the inflammatory process, e.g. cytokines and vascular adhesion molecules (VCAM), through various signalling pathways (Khan *et al.*, 2006). It has been suggested by results of *in vitro* studies that tea could inhibit the inflammatory factors (Sies *et al.*, 2005). However,

there is little evidence for anti-inflammatory effects of green tea consumption in randomised controlled human trials, and effects of tea on the inflammatory markers are not clearly understood. A four-week green tea supplementation study (daily consumption= 600ml) conducted among 20 smokers showed that the concentration of C-reactive protein (CRP), an inflammatory marker associated with an increased risk of cardiovascular diseases, decreased, but the decrease did not reach statistical significance (Lee *et al.*, 2005).

Anti-microbial effects:

The immune system is a powerful but complex defence system against infectious agents such as bacteria. This complex system involves interactions of different immune cells, e.g. T cells and B cells (Jain *et al.*, 2006). However, the immune system is often overcome, and pathogenic infections require treatment with antibiotics. The misuse of antibiotics and rapid microbial evolution has led to the development of antibiotic-resistance of bacteria, e.g. methicillin-resistant *Staphylococcus aureus* (MRSA), first reported in the 1960s (Jain *et al.*, 2006). MRSA is now a common cause of hospital-acquired infections, and community-acquired infections are increasing and a source of concern. A well-maintained immune system is essential to provide a stronger and more sufficient shield against infections. Some herbs and beverages, such as green tea or its catechins, have demonstrated an immunomodulatory effect and also have shown anti-microbial effects against pathogens (Cabrera *et al.*, 2006; Hamer, 2007; Lau, 2010). Catechins in green tea, especially EGCG, have been reported to inhibit the growth of several kinds of bacteria, e.g. *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* (Hamer, 2007). With the

combined use of green tea catechins and antibiotics, synergistic effects have been observed, converting the antibiotic-resistant strain into one which is more sensitive to antibiotics (Hamer, 2007; Lau, 2010). Though green tea catechins have been shown to be anti-microbial, the underlying mechanisms are not yet known.

Antioxidant and pro-oxidant paradox of green tea: attribution to the beneficial effects to health

Antioxidant effects:

Many natural phenolic-rich foods and beverages, including green tea, have been reported to combat oxidative stress, and prevent oxidative stress- associated diseases (Cabrera *et al.*, 2006; Moore *et al.*, 2009). This potential beneficial effect is considered to be, at least in part, attributed to their antioxidant properties (Benzie *et al.*, 1999b; Stevenson & Hurst, 2007). Catechins in green tea has been found to be able to quench and scavenge ROS, and can chelate oxidative metals, such as iron and copper, in *in vitro* and *in vivo* studies (Halliwell, 2007a; Stevenson & Hurst, 2007).

Numerous studies have demonstrated the antioxidant effects of green tea *in vitro*, and *in vivo*. Benzie *et al.* (1999b) demonstrated that a single-dose green tea consumption could increase the plasma total antioxidant status, as measured by the FRAP assay, within 40 minutes. Another group studying the correlation between green tea antioxidant capacity and tea concentrations and the extent of the antioxidant effect in humans has also shown a significant increase in plasma FRAP within an hour after single-dose green tea administration in a randomised, cross-over study (Pecorari *et al.*, 2010). Other studies of

regular consumption of green tea for a longer period of time have also shown antioxidant effects of green tea by means of effects on oxidative stress biomarkers. In a randomised, placebo-controlled, parallel study, 133 heavy smokers were randomised into one of three groups (decaffeinated green tea, decaffeinated black tea and water placebo) and required to consume 4 cups of tea or water a day for 4 months (Hakim *et al.*, 2003). It was reported that 4-cup daily intake of decaffeinated green tea for 4 months was associated with a significant decrease in urinary 8-oxodG (an oxidative stress marker) by 31% among heavy smokers (Hakim *et al.*, 2003). Erba *et al.* (2005) also reported that daily moderate green tea consumption (250mg catechins, or 1-2 cups tea per day) for 42 days could benefit antioxidant defence (by measuring total antioxidant activity in plasma), and protect lymphocytes from oxidative damage (assessed by using the comet assay after stressing cells with iron/ascorbic acid mixture) in 20 healthy humans (10 in green tea group and the others in control group). Our group has also demonstrated the genoprotective effect of green tea consumption in a placebo-controlled, multiple cross-over human supplementation study among 18 healthy volunteers (Han *et al.*, 2011). In this study, our group demonstrated that consumption of both Loongjin and screw-shape green teas at a concentration of 1%w/v (a dietary relevant dose) for 4 weeks (two cups a day) significantly decreased oxidation-induced DNA damage (assessed by the Fpg-assisted version of the comet assay to determine the level of oxidised purines) and increased the resistance of DNA against oxidant challenge (assessed by H_2O_2-stress model of the comet assay) in lymphocytes (Han *et al.*, 2011).

However, although some studies have shown a positive role of green tea against oxidative stress, it is undeniable that the ROS-generating property of green tea or green tea catechins has been observed and reported in *in vitro* models (Oikawa *et al*., 2003; Halliwell, 2006 & 2007; Aoshima, 2008; Lambert & Elias, 2010). This might be in contradiction with other human or animal studies showing the antioxidant effect of green tea. However, being pro-oxidant raises a possibility towards a more detailed, comprehensive mechanistic and protective role of green tea in humans.

Pro-oxidant effects:

Catechins belong to the family of polyphenols which oxidise rapidly in culture medium, and generate hydrogen peroxide and other pro-oxidants, such as quinones (Chai *et al.*, 2003; Halliwell, 2007b; Aoshima, 2008; Lambert & Elias, 2010). The generation of hydrogen peroxide has been reported in several polyphenol-rich beverages, and green tea has been shown to produce H_2O_2 at a level over 100mM in a time- and dose- dependent manner in the presence of metal ions, e.g. Cu (II) (Akagawa *et al.*, 2003). The generation of hydrogen peroxide was observed to reach a plateau after 12-hours' incubation at room temperature, and a higher temperature was found to be a contributing factor for an increased generation of hydrogen peroxide (Akagawa *et al.*, 2003; Aoshima & Ayabe, 2007). Chai *et al.* (2003) demonstrated that green tea was able to generate hydrogen peroxide in culture medium (Dulbecco's modified Eagle's medium, DMEM), as measured by ferrous ion oxidation- xylenol orange (FOX) assay, and the generation of hydrogen peroxide was found to increase with the concentration of green tea. The same group showed that a higher green tea concentration resulted in lower viability of PC12

cells, and this effect was eliminated by the addition of catalase, showing that the cytotoxicity is due to the generation of hydrogen peroxide. This was supported by another *in vitro* study conducted by Oikawa *et al.* (2003) on a human leukaemia cell line, reporting that catechins can generate ROS, such as hydroxyl radicals, in addition to hydrogen peroxide. The group has also demonstrated that addition of catechins can result in the increase in 8-oxodG (an oxidative stress marker) and caused DNA fragmentation through Cu (II)/Cu (I) redox mechanism. Though results from *in vitro* studies showing the pro-oxidant role of green tea seem to contradict a beneficial role for green tea, no epidemiological studies or even human intervention studies have provided evidence or have been conclusive in the role of green tea inducing oxidative stress. However, very high doses of green tea extract have been shown to induce liver damage in cell and animal models, and in human subjects (Schmidt *et al.*, 2005; Chacko *et al.*, 2010; Wang *et al.*, 2010). Therefore, more effort has to be paid to investigating the antioxidant or pro-oxidant role that green tea polyphenols play *in vivo*, possibly focusing on mechanisms that could be elicited by oxidant challenge. Consequently, it cannot be assumed that very high doses of green tea are safe.

Summary:

Green tea is rich in polyphenols, particularly catechins, which have powerful antioxidant properties but low bioavailability. Though catechins in green tea may not be highly bioavailable, the consumption of green tea has been associated with decreased risk of many age-related diseases. These diseases have been suggested to be a result of increased oxidative stress, and so the potential benefit of green tea, though no conclusive results

exist at present, is thought to be due to its antioxidant property. Several human intervention and animal studies have shown that green tea or green tea catechins can lower oxidative stress. Paradoxically, some *in vitro* studies using green tea have shown that it is a pro-oxidant rather than an antioxidant, and could potentially increase oxidative stress. A theory which may be able to link these two opposing observations together is that this pro-oxidant effect may not be damaging but modulatory due to its mildness and consistency (when an antioxidant food is consumed regularly and when bioavailability is low), this in turn enables the antioxidant defence system to be more effective to a later, more severe oxidative challenge. This concept is explored in the following sections.

Possible Mechanism of Green Tea *in vivo* in Relation to its Pro-oxidant Property, with Particular Focus on the Nrf2/ARE Signalling Pathway

Introduction:

In the past decade, several lines of evidence have shown that production of major cytoprotective elements is regulated by an antioxidant response element (ARE), also known as electrophile response element (EpRE), the induction of which is mediated by the nuclear transcription factor erythroid 2p45 (NF-E2)-related factor 2 (NFE2L2, but more commonly referred to in the literature as Nrf2) in *in vitro* and animal studies. The redox-sensitive gene promoter region of the ARE responds to a pro-oxidant change in redox tone and upregulates genes that control various cytoprotective mechanisms or agents, particularly Phase II detoxification, antioxidant enzymes, haem oxygenase and DNA repair enzymes (Surh *et al*., 2008). The products of these genes include haem oxygenase-1 (HO-1), superoxide dismutase (SOD), glutathione (GSH), and DNA repair enzymes, including human 8-oxoguanine DNA glycosylase (hOGG1) (Halliwell & Gutteridge, 2007; Surh *et al.,* 2008; Zhang *et al.,* 2010). Some plant-based antioxidants, such as catechins in green tea, have been shown to act as pro-oxidants under some circumstances *in vitro*, generating hydrogen peroxide (Halliwell & Gutteridge, 2007). Hydrogen peroxide has long been regarded as a deleterious molecule to cells. However, in the last decade, its possible involvement in regulating signalling pathways for adaptive responses, e.g. survival, has received attention, although it is not known if the generation of hydrogen peroxide from tea catechins occurs *in vivo* (in humans). There is a possibility

that a dietary 'antioxidant' could induce a mild pro-oxidant effect on cellular redox balance that could cause the cell to produce cytoprotective elements that could, for example, increase DNA repair, and/or upregulate endogenous antioxidants, through the activation of the Nrf2-ARE signalling pathway (Benzie & Wachtel-Galor, 2010).

Generation of hydrogen peroxide in green tea: a candidate molecule to trigger cellular responses for indirect cytoprotection

Green tea has been demonstrated to generate hydrogen peroxide *in vitro* (Chai *et al.*, 2003; Halliwell, 2007b). Hydrogen peroxide is a known damaging molecule to cells, posing a pro-oxidant hazard to proteins, lipids and DNA (Holbrook & Ikeyama, 2002; Kothen & Nyska, 2002; Halliwell & Gutteridge, 2007). However, in the past decade, the view that hydrogen peroxide is always unwelcome has been challenged, as several lines of evidence have shown another role of hydrogen peroxide in signal transduction for cellular responses, including survival, which is crucial for maintaining proper development and proliferation of cells (Veal *et al*., 2007; Bao *et al*., 2009; Wu *et al*., 2010; Bae *et al*., 2011; Gough & Cotter, 2011).

Hydrogen peroxide as a secondary messenger for cellular signalling:

Hydrogen peroxide contributes to oxidative stress, playing a role in the pro-oxidant side of redox balance. Although having less oxidizing power, hydrogen peroxide was thought to be more damaging compared to superoxide since hydrogen peroxide is relatively stable with longer half-life of approximately 10^{-5} seconds, whereas that of superoxide is 10^{-6} seconds and, unlike the superoxide anion, hydrogen peroxide can easily diffuse within

cells (Giorgio *et al*., 2007; Mishina *et al*., 2011). The major production site of ROS is the mitochondrial respiratory chain (Reth, 2002; Giorgio *et al*., 2007; Finkel, 2011). Electrons escape from the electron transport chain during aerobic respiration, resulting in incomplete reduction of oxygen to form superoxide, which will generate hydrogen peroxide with the aid of manganese superoxide dismutase. In addition to the leakage of electrons from the electron transport chain, it has been found that the activation of NAPDH oxidases (Nox) family leads to the formation of superoxide which eventually gives rise to the generation of hydrogen peroxide (Bao *et al*., 2009; Wu *et al*., 2010; Gough & Cotter, 2011). This was first thought to occur only in phagocytic immune cells, however, the generation of ROS via the activation of the Nox family has also been shown in non-immune cell types, including human fibroblasts (Reth, 2002; Forman, 2007; Lambeth *et al*., 2007; Veal *et al*., 2007; Finkel, 2011).

Most of the studies have shown the cell-damaging role of hydrogen peroxide, resulting in stressed conditions and promoting ageing, apoptosis and necrosis. However, there have been controversial studies demonstrating hydrogen peroxide can mimic the action of growth factors and showing the mitogenic effects of hydrogen peroxide (Reth, 2002; Veal *et al*., 2007). This leads to the further investigation into the multifactorial role of hydrogen peroxide, and it is now recognised to be a ubiquitous intracellular secondary messenger involved in regulating cellular signalling under sub-toxic or physiological conditions (Reth, 2002; Veal *et al*., 2007). Hydrogen peroxide is a small molecule which is rapidly generated by Nox family near plasma membranes or by mitochondria upon stimulation and it is easily eliminated by endogenous antioxidants and enzymes. It

diffuses locally within a cell to react with specific cellular targets, such as the cysteine residues of proteins (Rhee *et al*., 2005; Bae *et al*., 2011; Finkel, 2011; Malinouski *et al*., 2011). It was thought that hydrogen peroxide might be able to diffuse across cells freely across plasma membranes due to its neutrality, but it has been demonstrated that hydrogen peroxide is not freely diffusible but is transported through special transporter proteins, known as aquaporin-3, and this limits hydrogen peroxide to signal locally within a cell rather than to diffuse out of the cells (Bienert *et al*., 2007; Miller *et al*., 2010; Mishina *et al*., 2011)

In contrast to other ROS, hydrogen peroxide is a relatively mild oxidant which specifically targets cysteine residues of various proteins for signalling (Bao *et al*., 2009; Wu *et al*., 2010; Bae *et al*., 2011; Finkel, 2011; Gough & Cotter, 2011). In general, there are four oxidation states of cysteine residues, they are disulphide (-S-S-), sulphenic acid (-SOH), sulphinic acid ($-SO_2H$), and sulphonic acid ($-SO_3H$). Formation of sulphinic acid and sulphonic acid is irreversible under physiological conditions, requiring robust oxidants (Veal *et al*., 2007; Forman *et al*., 2010; Bae *et al*., 2011). With a mild oxidant such as hydrogen peroxide, the –SH group of a cysteine residue can be oxidised to give sulphenic acid which can also be re-reduced by endogenous antioxidants or enzymes, e.g. glutathione and thioredoxins. Only those deprotonated cysteine residues existing in the form of a cysteine thiolate anion ($-S^-$) having a lower pKa at neutral pH, are targeted for oxidisation (Veal *et al*., 2007; Forman *et al*., 2010; Bae *et al*., 2011). Proteins with deprotonated cysteine residues include protein tyrosine phosphatases (PTP) consisting of cysteine residues appearing as thiolate anions at the active motifs. PTP1B is a well-

demonstrated example of a redox-regulated protein (Mueller *et al*., 2008; Brandes *et al*., 2009; Tanner *et al*., 2011). It has been shown that cysteine residues of PTP1B are oxidised by hydrogen peroxide giving rise to the formation of a sulphenic acid intermediate, thereby inhibiting the activity of PTP1B (Lee *et al*., 1998). An increase in ROS levels has also been shown to inhibit PTP activity (Meng *et al*., 2004). The oxidation was subsequently reversed by endogenous enzymes such as peroxidases and GSH (Lee *et al*., 1998). Apart from PTPs, the reversible oxidation of deprotonated cysteine residues can also be found in protein kinases, ion channels and transcription factors. Accumulating evidence has shown that cellular antioxidant defence is regulated by intracellular ROS levels, and one well established example is the transcription factor Nrf2, which binds to the ARE, which in turn regulates the expression of a battery of antioxidant and detoxifying genes (Benzie & Wachtel-Galor, 2010; Zhang *et al*., 2010).

Nrf2-ARE signalling mechanism: a close relationship with redox tone

Nrf2 is a transcription factor characterised by its basic leucine zipper (bZIP) DNA binding domain which enables Nrf2 to recognise and bind to the ARE (Lee & Johnson, 2004; Calabrese *et al.*, 2009). It is known to be a crucial regulator of antioxidant response, and thereby controlling the expression of stress-responsive and cytoprotective proteins (Lee & Johnson, 2004; Eggler *et al.*, 2008; Benzie & Wachtel-Galor, 2010; Zhang *et al*., 2010). This 66-kDa transcription factor has six highly conserved domains, each with different functions, including the bZIP for promoter region recognition, recruitment of co-activators, and interaction with other regulatory proteins (Lee & Johnson, 2004).

Under inactive conditions (resting), Nrf2 is present in cytoplasm bound to a cysteine-rich regulatory protein, known as Kelch-like ECH-associated protein 1 (Keap1). Keap1 is in turn bound to the Cullin3-based E3-ubiquitin ligase complex to enable ubiquitination of Nrf2 followed by proteosomal degradation. This mechanism keeps a low level of Nrf2 in the cytoplasm, and ensures a short half-life of around 10-20 minutes (Kundu & Surh, 2009). Keap1 is sensitive to changes in cellular redox tone due to its cysteine-rich residues and the reversible modification of thiol groups by oxidation (Benzie & Wachtel-Galor, 2010). Once there is a pro-oxidant change in cellular redox tone (active conditions), e.g. due to exposure to pro-oxidants or electrophiles, Nrf2 is dissociated from the thiol group oxidised-Keap1, and this allows Nrf2 to escape ubiquitination and degradation (Benzie & Wachtel-Galor, 2010). Thiol groups are subject to oxidation, interfering with the function of protein (inactive form). Among the 27 thiol groups present in Keap1, it has been reported that there are three critical cysteine groups controlling Nrf2-Keap1 interactions, i.e. Cys^{151} for Nrf2 activation, and Cys^{273} and Cys^{288} for Nrf2 suppression (Surh *et al.*, 2008; Yamamoto *et al*., 2008). The resulting Nrf2 dissociated from Keap1 is, therefore, stabilised in cytoplasm without subsequent proteosomal degradation, leading to nuclear translocation and accumulation (Figure 1.5) (Benzie & Wachtel-Galor, 2010). However, it has been shown that the oxidative modification of cysteine residues in Keap1 was not completely responsible for the dissociation of Nrf2, i.e. the modification did not seem sufficient enough to disrupt the interactions between Keap1 and Nrf2 (Eggler *et al*., 2005). Apart from the covalent and oxidative modification of thiol groups of Keap1, protein kinases involved in other signalling pathways have also been reported to aid the dissociation of Nrf2 from Keap1,

stabilisation and nuclear translocation of Nrf2 by phosphorylation of Nrf2 on its serine and threonine residues (Figure 1.5) (Surh *et al.*, 2008). Those protein kinases include protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinases (MAPK) (Owuor & Kong, 2002; Surh *et al.*, 2008; Benzie & Wachtel-Galor, 2010). Another study investigating the redox tone affecting the interaction of Nrf2 and Keap1 has revealed the presence of evolutionary conserved cysteine residues in Nrf2 and their role in regulation the activity of Nrf2 (He & Ma, 2009). The group has revealed that cysteine residues in Nrf2 were conserved among various species and these residues have been shown to interact with an arsenic-based fluorophore and phenylarsine oxide (PAO) (He & Ma, 2009). Several cysteine residues have also been identified to be involved in redox signalling. Using deletion and mutational analyses, it was found they are mainly responsible for regulating the inducer binding (Cys^{119}, Cys^{191}, Cys^{235}, Cys^{311}, Cys^{316}, Cys^{414} and Cys^{506}), and nuclear localisation of Nrf2 (Cys^{199}) (He & Ma, 2009). He and Ma (2009) have, therefore, suggested a dual sensing relationship, in which cysteine residues are present in both Nrf2 and Keap1 to regulate the Keap1-Nrf2-ARE signalling cascade. Upon the oxidative modification of cysteine residues to activate Nrf2, Nrf2 will thus be released and moved into the nucleus for ARE activation.

With its accumulation in the nucleus and the presence of bZIP DNA binding domain, Nrf2 recognises and binds to the ARE in association with another small nuclear transcription factor Maf, replacing the transcription repressor Bach1 and thereby activating the ARE. Bach1 (BTB and CNC homology 1, basic leucine zipper transcription factor 1) is a transcription repressor that is ubiquitously expressed in tissues

(Kaspar & Jaiswal, 2010). It, in association with Maf, binds to the ARE and represses the induction of ARE-driven cytoprotective gene expression without pro-oxidant challenge (Kaspar & Jaiswal, 2010). As noted, the ARE is a redox sensitive gene promoter region, which regulates the transcription of Phase II detoxification enzymes, and other cytoprotective products for antioxidant defence, e.g. haem oxygenase-1 (HO-1) and NAD(P)H:quinone-oxidoreductase-1 (NQO1) (key products of ARE and products that contain ARE in their promoter regions are summarised in Table 3) (Halliwell & Gutteridge, 2007; Benzie & Wachtel-Galor, 2010). With the activation of the Nrf2-ARE signalling pathway, cellular redox homeostasis can be restored and maintained, and further oxidative damage to cells can be minimised (Lee & Johnson, 2004).

Figure 1.5 The stabilisation and phosphorylation of Nrf2 in the Nrf2/ARE signalling pathway (adapted from Surh *et al.*, 2008)

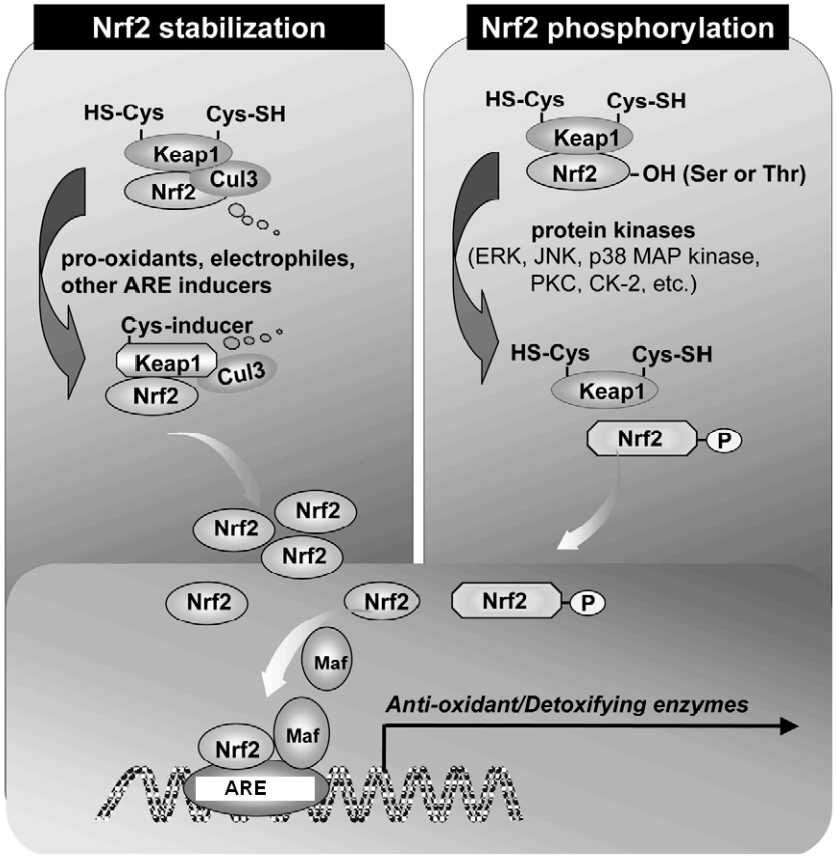


Table 1.3 Products that are regulated via the Nrf2-ARE signalling pathway (Halliwell & Gutteridge, 2007); those marked with * are of particular interest in this study

Key products	**Functions**
Haem oxygenase-1 (HO-1)*	To metabolise haem to generate iron, biliverdin and carbon monoxide. Biliverdin is subsequently converted by biliverdin reductase to bilirubin, an endogenous antioxidant which can scavenge lipid peroxyl radicals; whereas carbon monoxide can inhibit platelet aggregation and act as a vasodilator.
NAD(P)H:quinone oxidoreductases (NQO-1)	To compete with one-electron reduction of quinone by cytochrome P450, which may generate ROS. By its two-electron reduction and detoxification of quinones and its derivatives, comparatively stable hydroquinone, which is removed by conjugation with GSH, will be produced.
Glutathione S-transferase (GST)	To reduce GSH conjugated with xenobiotics
Glutamate cysteine ligase catalytic and modulatory subunits (GCLC/GCLM)	To form glutamate-cysteine ligase (GCL) involved in GSH synthesis. GCLC is a catalytic subunit to control the enzymatic activity of GCL; while GCLM controls the activity and binding affinity of GCLC towards substrates.
Ferritin	To chelate free iron and store it in the form of Fe (II) ions, which will then be oxidised to Fe (III) with the release of hydrogen peroxide As endogenous antioxidants work in a network, the production of hydrogen peroxide can be removed by GPx or CAT.
Superoxide dismutase (SOD)	To catalyse the conversion of superoxide to oxygen and hydrogen peroxide, and act as a primary endogenous enzyme antioxidant.

Human 8-oxoguanine DNA glycosylase (hOGG1)*	To act as a primary DNA repair enzyme (base excision repair) for 8-oxo-7,8-dihydroguanine (8-oxoGua), an oxidised purine.

Nrf2-ARE signalling mechanism: phytochemical induction in *in vitro* and *in vivo* studies

The Nrf2-ARE signalling pathway is known to be a crucial stress-sensitive signalling pathway, maintaining the cellular redox balance and lowering or opposing oxidative damage by controlling the expression of downstream cytoprotective products (Eggler *et al.*, 2008; Benzie & Wachtel-Galor, 2010; Zhang *et al.*, 2010). Some phytochemicals, i.e. plant-based chemicals such as dietary antioxidants, have been shown to be able to induce this pathway, and are regarded as "ARE inducers" (Benzie & Wachtel-Galor, 2010; Zhang *et al.*, 2010). Interestingly, some of these phytochemicals are involved in pro-oxidant actions *in vitro* (though this is not yet confirmed *in vivo*), and these include catechins in green tea, resveratrol from grapes and quercetin from onions (Kundu & Surh, 2009; Zhang *et al.*, 2010). Although these phytochemicals have been suggested to be pro-oxidants *in vitro*, their pro-oxidant activity is suggested to be weak in nature, and insufficient to cause severe oxidative damage (Zhang *et al.*, 2010). As the Nrf2-ARE signalling pathway is sensitive to pro-oxidant change in redox tone in cells, it is possible that these ARE inducers may lead to a continuous or intermittent, mild oxidative stress to cells, triggering the Nrf2-ARE signalling pathway and promoting the upregulation of cytoprotective products to protect target molecules from severe oxidative damage (Zhang *et al.*, 2010).

Most of the previous studies conducted on phytochemical induction of the Nrf2-ARE signalling pathway have been *in vitro* and animal studies, with particular interest in the involvement in cancer prevention (Eggler *et al.*, 2008; Gopalakrishnan & Kong, 2008). Using these tools, phytochemicals have been reported to be able to modulate the expression of various Nrf2-ARE mediated cytoprotective gene products, e.g. glutathione-S-transferase (GST), GPx, GSH, and haem oxygenase-1 (HO-1) (Lee & Johnson, 2004; Kobayashi & Yamamoto, 2006).

Curcumin, a diferuloylmethane from rhizomes of tumeric, was demonstrated to elevate the ARE-mediated expression of HO-1 and GCLM expression in cultured human monocytes at gene and protein levels, after curcumin incubation for 4 hours and for 8 hours respectively, by the upregulation of Nrf2 (Rushworth *et al*., 2006). In addition to HO-1 and GCLM, gene expression of other ARE-mediated products, such as NQO1 and ferritin, were shown to be elevated after 4-hour incubation of curcumin (Rushworth *et al*., 2006). This was accompanied by increased nuclear accumulation and ARE binding of Nrf2, which was observed 30 minutes after curcumin incubation (Rushworth *et al*., 2006). Resveratrol, a bioactive ingredient found in grapes, was shown to enhance the restoration of cigarette smoke extract (CSE)-induced depletion of cellular GSH (an endogenous antioxidant) via enhanced nuclear translocation and accumulation of Nrf2 using human primary lung epithelial A549 cells in an *in vitro* model (Kode *et al.*, 2008). Interestingly, it was observed that nuclear translocation of Nrf2 occurred within an hour after CSE exposure, but was not seen after a longer exposure time, i.e. 4-24 hours (Kode *et al.*, 2008). This may be due to the effects of aldehyde present in CSE on modulation of

sulphydryl groups of Keap1-Nrf2, resulting in subsequent failure of Nrf2 nuclear translocation after the 4th hour (Kode *et al.*, 2008). This also implies a possibility that long-term intense oxidative or electrophilic stress in cells may lead to an inactivation of the Nrf2-ARE signalling pathway, which may result in a lack of cytoprotection. A hydroxylated analog of resveratrol, piceatannol, was demonstrated to enhance the gene and protein expression of HO-1 in human breast epithelial MCF10A cells 3 hours and 6 hours, respectively, after incubation with piceatannol of 30μM (Lee *et al*., 2010). The enhancement was accompanied with increased nuclear translocation of Nrf2 followed by increased ARE binding, and the effects were eliminated by silencing of Nrf2 using siRNA (Lee *et al*., 2010). Indeed, Lee and colleagues (2010) have speculated the catechol group of piceatannol was responsible for the observed effects by interacting with critical cysteine residues of Keap1 in cytosol which helps the dissociation of Nrf2 or by activating the upstream effectors of Nrf2 such as PKC. In another *in vitro* study using hepatoblastoma HepG2 cells, quercetin, a promising polyphenolic compound in decreasing oxidative stress, induced Nrf2 expression at both transcriptional and translational levels, activating the downstream NQO1 (NADPH:quinine oxidoreductase) expression, a cytoprotective gene mediated by the ARE, possibly via the stabilisation of Nrf2 protein and modification of cytosolic Keap1 (Tanigawa *et al.*, 2007).

Curcumin, as reported from several animal studies, has been shown to promote the nuclear translocation of Nrf2 which binds to ARE, and further to upregulate HO-1, GST and NQO1 expression at the mRNA and protein level (Farombi *et al.*, 2008; Garg *et al.*, 2008). Increased nuclear accumulation and ARE binding of Nrf2 in a time-dependent

manner has been observed in albino rats after gavaging curcumin at 200mg/kg for 4 days, accompanied with increase in the protein expression and activity of HO-1 (Farombi *et al*., 2008). This study also demonstrated the protective role of curcumin against dimethylnitrosamine-induced liver injury (Farombi *et al*., 2008). The results were in agreement with a similar study investigating the modulatory effects of curcumin against carcinogen exposure using male Swiss albino mice of 6-8 weeks old (Garg *et al*., 2008). In addition to resveratrol, quercetin and curcumin, other phytochemicals, mostly phenolic and sulphide-containing compounds, are also found to be "ARE inducers". These include diallyl sulphide in garlic and genistein in soy products (Zhang *et al.*, 2010).

Nrf2-ARE signalling mechanism: evidence of green tea as an ARE inducer in *in vitro* and animal studies

Accumulating evidence shows that phenolic compounds exert their protective effects on cells by stimulating the Phase II detoxification and antioxidant enzymes via Nrf2-ARE signalling pathways (Khan *et al.*, 2006). Green tea, the focus of this current project, is a polyphenol-rich beverage, and has shown its effects on modulating the Nrf2-ARE signalling pathway in many cell culture and animal studies (Wu *et al.*, 2006; Ogborne *et al.*, 2008; Sahin *et al*., 2010). Among the four major bioactive catechins in green tea, EGCG has demonstrated its prominent effect on Nrf2 nuclear translocation, induction of ARE-luciferase reporter gene transactivation, and possible mechanism in initiating Nrf2-ARE signalling pathway (Andreadi *et al.*, 2006; Wu *et al.*, 2006; Na *et al.*, 2008). EGCG has been reported to enhance the mRNA expression of the haem oxygenase-1 gene (*HMOX-1*), GCLC (which regulates GSH synthesis), and MnSOD in a concentration-

dependent manner (25-100μM) in mammary epithelial MCF10A cells (Na *et al.*, 2008). These enhancements of mRNA expression were shown to be mediated by binding of Nrf2 on ARE, in which Nrf2 binding activity was concentration- and time- dependent on EGCG with increasing Nrf2 nuclear accumulation observed (Na *et al.*, 2008). In the same study, EGCG was found to increase phosphorylation of ERK1/2 and Akt with time, and the use of inhibitors of MEK and PI3K (which regulate the phosphorylation of ERK1/2 and Akt) inhibited the EGCG-induced Nrf2 nuclear translocation (Na *et al.*, 2008). This finding provides evidence for the involvement of MEK-ERK1/2 and PI3K-Akt in activating the Nrf2 and its corresponding ARE-controlled cytoprotective genes and products, i.e. *HMOX-1* and MnSOD, in response to EGCG treatment. A study by Wu *et al.* (2006) showed that PI3K-Akt was a major pathway for EGCG to trigger HO-1 expression compared to MEK-ERK1/2 due to its complete abrogation of HO-1 protein expression with the PI3K-Akt pathway inhibited. Although *HMOX-1* mRNA expression was shown to be upregulated, HO-1 protein expression was not seen to be enhanced by 15μM EGCG in another study conducted by Andreadi *et al.* (2006) using a breast epithelial MDA-MB468 cell model. This may indicate that incubation time or concentrations of EGCG applied on cells are critical, or the effect of EGCG is cell-line/type specific. According to Wu and colleagues (2006), upregulation of EGCG-induced HO-1 protein expression was seen to be concentration- and time- dependent (25-100μM) in bovine aortic endothelial cells, and this observed upregulation was in line with increased *HMOX-1* mRNA expression reported by Na *et al.* (2008). Wu *et al.* (2006) also demonstrated the ability of EC, another major catechin in green tea, at concentrations of 50 and 100μM, to induce HO-1 protein expression. This increase in expression due to EC,

however, was not as potent as that of EGCG. No change in expression was observed when the other two catechins, i.e. EGC and ECG were applied, which was not in agreement with the findings of Ogborne *et al.* (2008) using the human monocytic leukaemia THP-1 cell line. The group showed that EGC, a relatively more bioavailable catechin compared to EGCG, at concentrations of 12.5, 50 and 100μM can upregulate HO-1 expression at transcriptional and translational levels (Ogborne *et al.*, 2008). HO-1 expression was found to be mediated by Nrf2 activation regulated by protein kinase C δ. The observed deviation may be due to the various cell line models applied, bovine aortic endothelial cells in Wu's group (2006) and human monocytic leukaemia THP-1 cell line in Ogborne's group (2008). In addition, the concentrations of catechins applied to cells were relatively high when compared to the small (nanomolar) amounts of catechins achieved in human plasma after drinking green tea, and this may also contribute to the deviation (Del Rio *et al.*, 2010). Although hydrogen peroxide may not be generated in a nanomolar range, redox balance may still be shifted to trigger the Nrf2 activation. EC has also been reported to be involved in preventing stroke damage through the activation of Nrf2/HO-1 pathway *in vitro* and *in vivo* (Shah *et al.*, 2010). EC was found to enhance HO-1 protein expression at 6 hours and Nrf2 nuclear translocation at 30 minutes *in vitro*, but this observation was not seen in HO-$1^{-/-}$ and Nrf$2^{-/-}$ mice (Shah *et al.*, 2010).

In summary, ARE induction is a sign of a pro-oxidant change in redox tone – a mild oxidative stress. Many phytochemicals have been reported to be "ARE inducers" which can induce the activation of the Nrf2-ARE signalling pathway. Particularly, with the increasing evidence shown in animal and cell culture studies and the pro-oxidant effects

of green tea observed *in vitro*, it is possible that cytoprotection attributed to the activation of Nrf2-ARE signalling pathway may be one of the mechanisms that mediate reported health benefits of green tea against oxidative stress and age-related diseases. One of the key effects of ARE induction is expression of *HMOX-1* gene and increased production of the HO-1 protein; another is increased production of hOGG1, an enzyme that catalyses the first step in the base excision repair (BER) pathway of DNA damage.

Nrf2-ARE signalling mechanism: Haem oxygenase-1

Haem oxygenase exists as two major isoenzymes in humans, haem oxygenase-1 (HO-1) and haem oxygenase-2 (HO-2) (Deshane *et al.*, 2005; Soares & Bach, 2008). Haem oxygenase-1, encoded by *HMOX-1*, is a stress responsive isoform of the haem oxygenase family. HO-1 is an inducible enzyme produced in response to stress and stimuli such as its substrate, i.e. haem level, heat shock, and UV light, and is mainly induced in liver, spleen, kidney, and the central nervous system (Exner *et al.*, 2004; Soares & Bach, 2008). HO-2, by contrast, is a constitutive enzyme widely expressed throughout tissues (Shibahara, 2003; Soares & Bach, 2008). Both HO-1 and HO-2 are catalytically active, and share 43% similarity in terms of amino acid sequence (Immenschuh & Ramadori, 2000; Shibahara, 2003). Although deficiencies of both HO-1 and HO-2 have been reported to be involved in various diseases, e.g. cardiovascular diseases, neurodegenerative diseases and renal diseases, being inducible and one of the key products of redox-sensitive Nrf2-ARE signalling pathway, HO-1 is an investigative tool for 'sensing' ARE activation and detecting a pro-oxidant change in redox tone (Shibahara,

2003; Exner *et al.*, 2004).

HO-1 is expressed transcriptionally and translationally in response to the oxidative-stress activated Nrf2-ARE signalling pathway, acting as a signature of oxidative stress. It is an essential but rate-determining enzyme regulating haem metabolism (Immenschuh & Ramadori, 2000; Otterbein *et al.*, 2003; Calabrese *et al.*, 2009). HO-1 catabolises haem released from different haemoproteins, e.g. haemoglobin, to ferrous ions (Fe^{2+}), carbon monoxide (CO) and biliverdin (which is further reduced to bilirubin with the aid of biliverdin reductase; Figure 1.6) (Immenschuh & Ramadori, 2000; Otterbein *et al.*, 2003; Calabrese *et al.*, 2009). HO-1 has demonstrated its cytoprotective effects in cell culture and animal models, and is regarded as an adaptive cellular defence mechanism (Immenschuh & Ramadori, 2000; Deshane *et al.*, 2005). In addition to the cytoprotective role of HO-1 in removing haem, the resulting haem catabolites have also been reported to provide protection, for example, *in vitro* and *in vivo* studies have shown that CO (at a concentration <250ppm) can resemble the role of NO which is beneficial to cardiovascular health by suppressing pro-inflammation responses, and biliverdin-derived bilirubin is an antioxidant, protecting cells against oxidative stress (Otterbein *et al.*, 2003; Shibahara, 2003). The other HO-1 product, iron, is known to be a pro-oxidant, and is thought to enhance the production of ROS production from the Fenton reaction (Balla *et al.*, 2005). However, upregulation in the expression of ferritin, an iron chelator, accompanies the increased HO-1 expression, thereby protecting cells from further oxidative damage (Balla *et al.*, 2005).

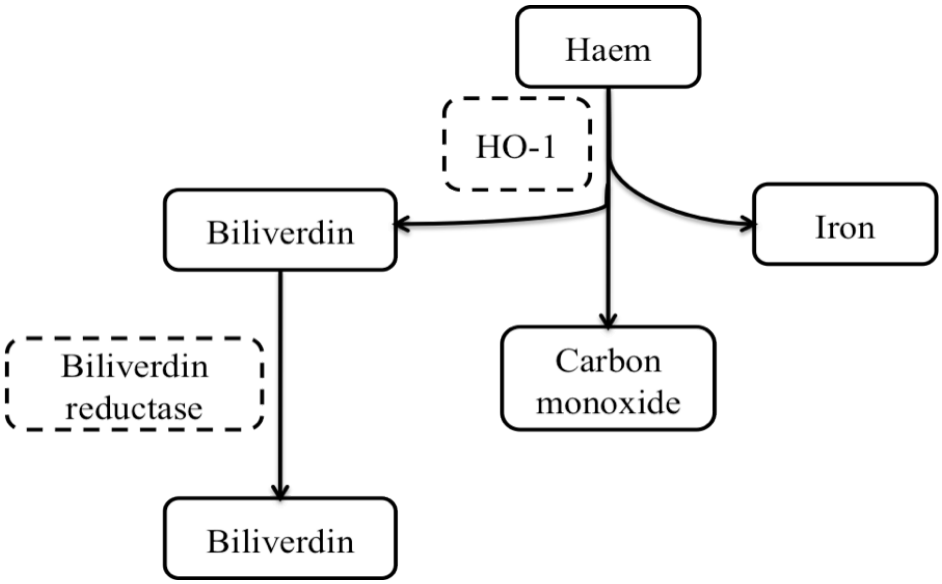


Figure 1.6 Catabolism of haem to biliverdin, iron and carbon monoxide through the action of HO-1

The cytoprotective role of HO-1 and its metabolites has contributed to alleviate the unfavourable conditions in various diseases, e.g. cardiovascular diseases, cancer, pulmonary diseases, inflammation and transplantation rejection (Exner *et al.*, 2003; Bella *et al.*, 2005; Deshane *et al.*, 2005). This has attracted wide attention for clinical applications. HO-1 has been hypothesised as a "therapeutic funnel" as HO-1 expression has been observed to play a critical role in the protective functions of several endogenous molecules used to suppress the progression of diseases (Figure 1.7) (Otterbein *et al.*, 2003; Bach, 2005; Calabrese *et al.*, 2009). Not only does it mediate the effects of these molecules, but also helps to amplify the protective effects, such as production of interleukin-10 (IL-10, an anti-inflammatory molecule) (Bach, 2005). IL-10 has been demonstrated to suppress the pro-inflammatory responses, and the actions of IL-10 have been associated with the increased expression and activity of HO-1 protein (Otterbein *et al.*, 2003; Soares & Bach, 2008). The resulting metabolite, CO, was shown to direct the

effects of IL-10, and also further to upregulate IL-10 production (Otterbein *et al.*, 2003; Soares & Bach, 2008). IL-10 has been thought to rely on HO-1 and resulting metabolite CO for its anti-inflammatory actions, and the loop cycle can help amplify the actions (Otterbein *et al.*, 2003; Soares & Bach, 2008). With inhibition of HO-1 expression, anti-inflammatory effects of IL-10 have been shown to be eliminated. However, the lost effects can be resumed by the administration of the either CO or HO-1 inducers (Otterbein *et al.*, 2003; Soares & Bach, 2008).

Inducers that help upregulate the expression of this cytoprotective but rate-determining HO-1 enzyme have thus been suggested to be therapeutically beneficial (Deshane *et al.*, 2003; Bach, 2005). Increasing evidence showing natural products, e.g. green tea polyphenols, can induce the expression of HO-1 protein expression and enzyme activity via the Nrf2-ARE signalling pathway is available from *in vitro* and animal studies, as mentioned previously (Wu *et al.*, 2006; Na & Surh, 2008; Ogborne *et al.*, 2008; Calabrese *et al.*, 2009; Pi *et al.*, 2010; Sahin *et al*., 2010; Zhang *et al.*, 2010). The potential health benefits observed in various human studies investigating green tea may possibly be, at least in part, due to the induction of HO-1 by drinking green tea.

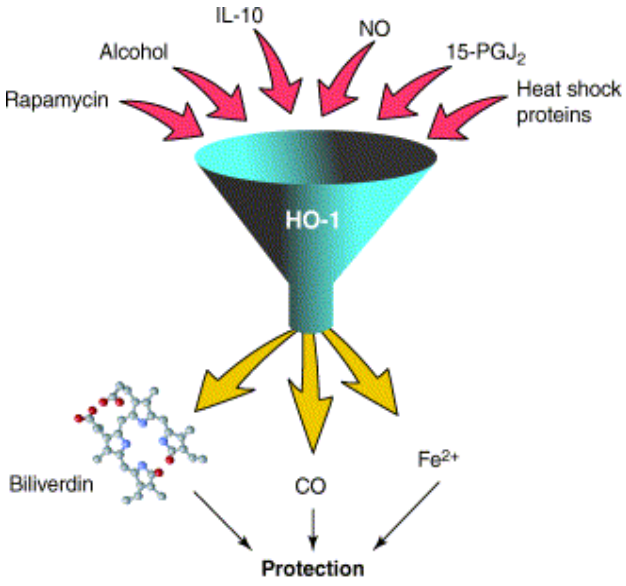


Figure 1.7 Haem oxygenase-1 acts as a "Therapeutic funnel", which mediates the functions of protective endogenous molecules and amplifies the effects (adapted from Otterbein et al., 2003)

Nrf2-ARE signalling mechanism: DNA repair via the human 8-oxoguanine DNA glycosylase 1 (hOGG1):

DNA is constantly challenged by ROS, causing oxidation-induced DNA damage (Klaunig *et al*., 2010). This damage can be either strand breaks or can give rise to oxidised lesions. Accumulated DNA damage has been implicated in various diseases, including cancer and Alzheimer's disease (Collins & Gaivao, 2007; Klaunig *et al*., 2010). DNA repair mechanisms have evolved in humans for the removal of the resulting oxidised lesions, and base excision repair (BER) is one of the most important pathways for repair (Figure 1.8) (Collins & Gaivao, 2007). BER is initiated by human 8-oxoguanine DNA glycosylase 1 (hOGG1), which is a human homologue of *E. coli.* formamidopyrimidine glycosylase (Fpg) which recognises and removes altered (oxidised)

bases (Klaunig *et al*., 2010). The protein hOGG1, encoded by the *OGG1* gene, exists in two major forms, i.e. α-hOGG1 and β-hOGG1 (Klungland & Bjelland, 2007). The former isoform works mainly in the nucleus whereas the latter functions in mitochondria (Klungland & Bjelland, 2007). With focus on nuclear DNA, hOGG1 is used here in this report to refer to its α-isoform. hOGG1 is known to be a bifunctional enzyme, which can act as both N-glycosylase or AP (apurinic/apyrimidinic) lyase, cleaving N-glycosidic bonds (which link base and sugar moiety of the backbone) and excising oxidised purines 7,8-dihydro-8-oxoguanine (8-oxoGua) (Collins & Gaivao, 2007; Frosina, 2007). Transcription factor binding sites for Nrf2 and AP-1 are located at the promoter region of *OGG1* gene, and the expression and activity of OGG1 may possibly be regulated by the oxidative challenge since the Nrf2-ARE signalling pathway is redox sensitive (Venugopal & Jaiswal, 1998; Giudice & Montella, 2006). It has been reported that the activation of the Nrf2-ARE pathway can enhance OGG1 expression (Giudice & Montella, 2006). Green tea, as a promising "ARE inducer" *in vitro*, may enhance the expression of DNA repair enzyme, which may be responsible for some of the reported health benefits of drinking green tea.

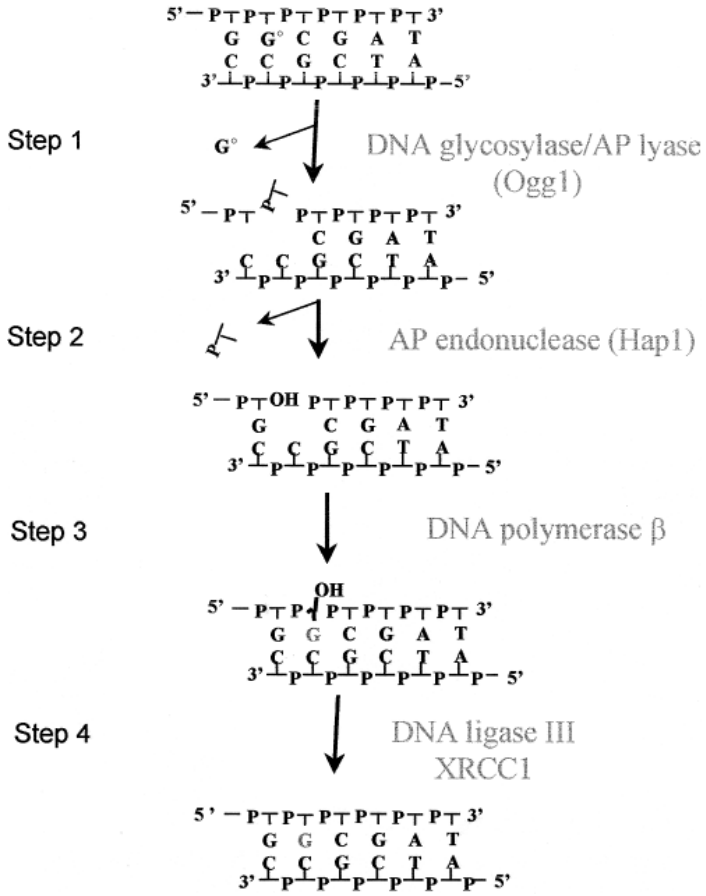


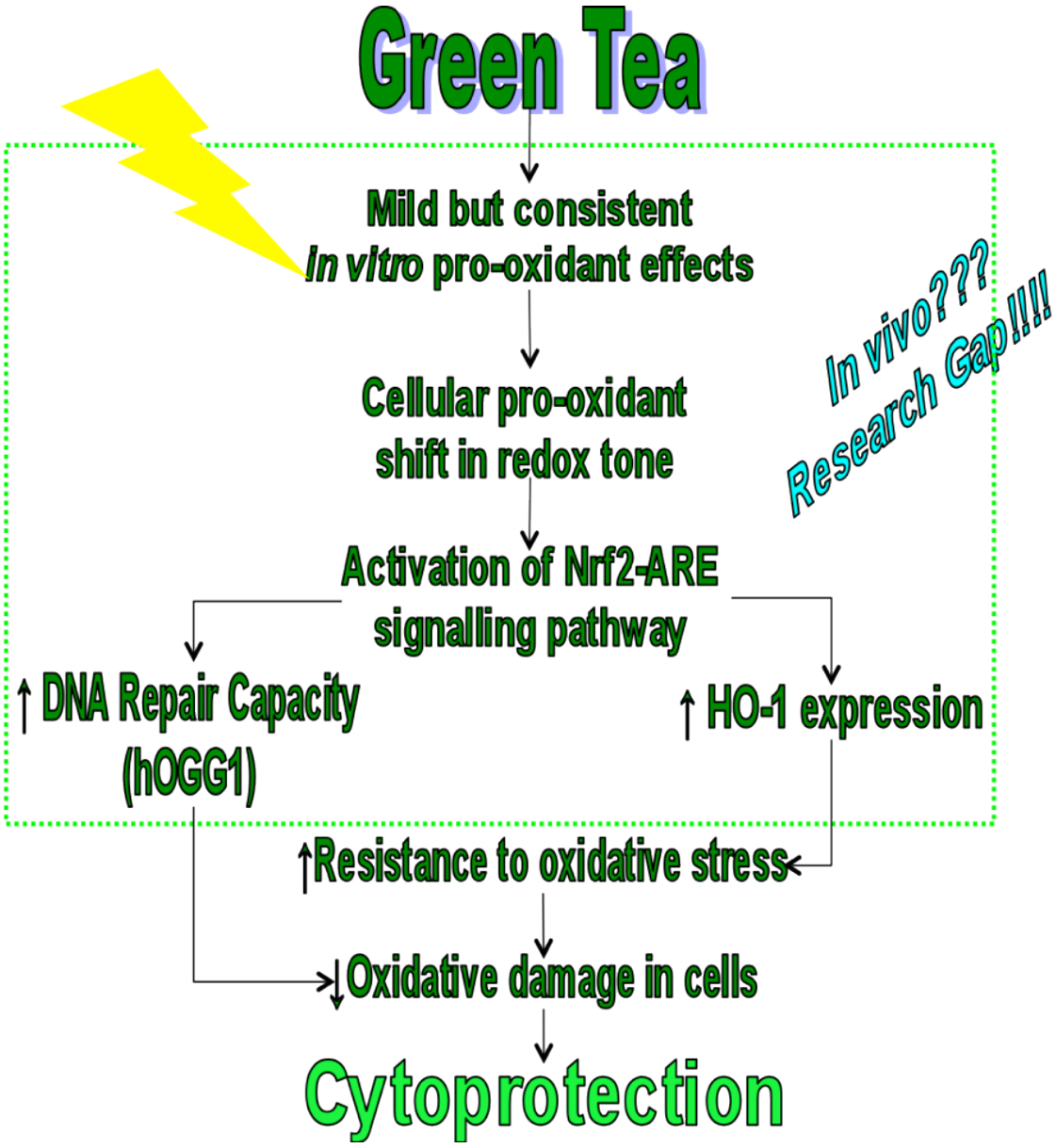
Figure 1.8 Base excision repair pathway (Baiteux & Radicella, 2000)

Summary and Research Needs:

There is a large and increasing burden of age-related disease in Hong Kong and all developed societies, and these age-related diseases are associated with increased oxidative stress. Oxidative stress is a change in redox balance in favour of pro-oxidants, and causes oxidation-induced damage to key biomolecules (DNA, lipids, proteins), and this damage affects the functions of molecules, organelles and cells. Green tea is a popular beverage in Asian countries, and has been reported to decrease the risk of age-related diseases. Our group has shown that green tea can decrease oxidative damage to DNA *in vitro* and *in vivo*. The effect is thought to be due to the high polyphenolic content, which makes up 35% dry weight of green tea. Catechins are the major polyphenols in green tea, at about 70% of total polyphenols, and have been reported to be responsible for the beneficial effects of green tea. Interestingly, green tea polyphenols can act as pro-oxidants *in vitro*. This observation may mean that simple, direct antioxidant action of green tea polyphenols is not the main or sole mechanism of protection, and thus more attention needs to be paid to other possible mechanisms, gene-nutrient interactions and effects on redox tone. A redox-sensitive gene promoter region, the ARE, which is activated by the binding of Nrf2, has been shown to regulate the production of major cytoprotective elements *in vitro* and in animal studies. This Nrf2-ARE signalling pathway responds to a pro-oxidant change in redox tone and upregulates genes that control the expression of various cytoprotective products. Accumulating evidence has shown that this Nrf2-ARE signalling pathway can be induced by natural products. With the potential pro-oxidant nature of green tea (though not yet confirmed *in vivo*), it is possible that green tea may also lead to an increased resistance to oxidative stress by acting as a

constant but mild inducer to trigger the Nrf2-ARE signalling pathway for cytoprotection involving, among others, increased HO-1 and hOGG1 (Figure 1.9). This knowledge would provide insights into the antioxidant:oxidant role of green tea, and the possible actions of green tea for potential health benefits. This is the focus of this study.

Figure 1.9 Concept map of the present planned study



Chapter 2
Aims, Objectives and Arrangement of the Thesis

Previous work by our group showed that regular intake of green tea (for 4 weeks) decreased oxidation-induced DNA damage in healthy middle-aged adults (Han *et al.*, 2011). However, it is not known whether this effect is an adaptive change to regular intake, or if it occurs quickly (in hours as opposed to days or weeks) after ingestion of green tea, nor are the mechanisms known. It is also not known if response is associated with the magnitude of change in the polyphenols levels in plasma after green tea ingestion. This present study was divided into two major parts, an *in vitro* study (using pooled human lymphocytes from volunteers) in which cells were exposed to green tea, and an *in vivo* study (in a controlled, single-blinded human intervention study) investigating acute response to a single dose and cellular adaptations after one week of regular intake of green tea.

The hypothesis of this study was that green tea can act as a mild pro-oxidant by generating hydrogen peroxide, and cause changes in redox balance, leading to the activation of the redox sensitive Nrf2/ARE signalling pathway for downstream cytoprotection. To test this hypothesis, this two-part study was performed, as follows:

1 – *In vitro* study:

Objectives:

1. To measure the concentrations of hydrogen peroxide generated in tea solutions at 37^{o}C
2. To investigate if green tea at low doses causes a change in:
    a. Oxidation-induced DNA damage in human lymphocytes (assessed using the Fpg-assisted comet assay)
    b. Gene expression of key players in the Nrf2/ARE signalling pathway, e.g. the indicator genes *HMOX-1*, *KEAP-1*, *BACH-1*, *NRF-2*, and also the DNA repair gene *hOGG1*.

If changes in *HMOX-1* were seen, an additional objective was:

3. To correlate *HMOX-1* expression with oxidation-induced DNA damage in human lymphocytes pre-incubated with green tea

2 – *In vivo* study

Objectives:

To investigate changes in key variables *in vivo* (key players in Nrf2/ARE signalling pathway and oxidation-induced DNA damage, as well as plasma polyphenols) in response to a single dose of green tea (acute response) in comparison to seven days' supplementation with green tea by examining if green tea supplementation:

1. Increases the gene and protein expression of key biochemical ARE products
2. Increases plasma antioxidant status

3. Decreases lymphocytic DNA damage
4. Increases DNA repair of oxidative lesions

In addition, the effects were examined in relation to changes in plasma tea polyphenolic levels at timed intervals after the single dose, and in fasting samples collected after 7 days of supplementation with green tea.

For readability, the following chapter, i.e. Chapter 3, covers the materials and methods used in both *in vitro* and *in vivo* studies. This chapter is followed by the description and results of the *in vitro* study (Chapter 4) and the *in vivo* supplementation study (Chapter 5). These chapters are written in the form of scientific papers, with brief background, necessary descriptions of methods if not given in detail already in Chapter 3, and examination of the results and implications. Chapter 6 is a summary of main findings, overall discussion, limitations and suggestions for future work, followed by concluding remarks.

Chapter 3
Materials and Methods

This chapter presents detailed information on methods used in both the *in vitro* (Chapter 4) and *in vivo* (Chapter 5) parts of this study.

Measurement of hydrogen peroxide generated in tea solutions:

Measurement of hydrogen peroxide (H_2O_2) was performed using a modified xylenol orange method (Jiang *et al*., 1992; Yuen & Benzie, 2003).

Reagent A was prepared from 25mM ammonium iron (III) sulphate-6-water ($NH_4Fe(SO_4)_2•6H_2O$) and 2.5M sulphuric acid (H_2SO_4), and reagent B was made up with 100mM sorbitol and 125µM xylenol orange in water. $NH_4Fe(SO_4)_2•6H_2O$, H_2SO_4, xylenol orange, 30% H_2O_2 solution and phosphate buffered saline (PBS) powder were all obtained from Sigma-Aldrich at molecular biology grade (St. Louis, MO, USA). Sorbitol was purchased from BDH Laboratory Supplies (Poole, England). A working reagent was mixed with reagents A and B in 1:100 ratio as required. To prepare the hydrogen peroxide standards, 10µl of 30% H_2O_2 were added to 8.8ml PBS to make up a stock solution of 10mM, and 80µl of this were transferred from the stock solution to another 10ml PBS to make up an 80µM H_2O_2 solution. This was used to prepare working H_2O_2 standards were prepared at concentrations of 5µM, 10µM, 20µM, 40µM, and 80µM in

PBS. Using a 96-well flat-bottomed microplate, 20µl of test sample (tea solutions before and after incubation) or standard were added along with 200µl working reagent solution into designated wells, and the mixture was incubated for 20 minutes at room temperature. The absorbance of the wells was then measured using a spectrophotometer (Benchmark Plus, Bio-Rad, Hercules, CA, USA) at a wavelength of 595nm.

Extraction and cryopreservation of lymphocytes:

An established protocol used in our laboratory was adopted for extraction and cryopreservation of lymphocytes from venous blood (described in detail in Han *et al*., 2011). Three milliliters of heparinised venous blood were transferred to a 15ml centrifuge tube and mixed gently with an equal volume of PBS. Three millilitres of Histopaque®-1077 were underlaid carefully at the bottom of the centrifuge tube, and the tube was centrifuged at 2000rpm for 20 minutes at 20^{o}C to isolate mononuclear cells (lymphocytes and monocytes) from whole blood. Since the majority of mononuclear cells in human blood consist of lymphocytes, the isolated cells will be referred to as lymphocytes in this thesis. The isolated lymphocytes (which appeared as a blurry milky layer in between the Histopaque®-1077 and plasma/PBS layers, as represented in Figure 3.1 were transferred to a clean centrifuge tube (Figure 3.1). The isolated lymphocytes were washed with cold PBS and centrifuged at 1200 rpm for 5 minutes at 4^{o}C. The supernatant was discarded. After a second wash, pelleted cells were re-suspended in 3ml freezing medium (FBS and DMSO in a ratio of 9:1), and were then aliquoted into 1.5ml eppendorf tubes. Each aliquot was frozen slowly in a thick walled polystyrene box at -80^{o}C for later use. As

needed, these cryopreserved cells were thawed quickly and washed twice with PBS just before use. A similar method was used to isolate lymphocytes from the buffy coat layer of previously centrifuged blood at 2000rpm for 10 minutes at 4^{o}C. The above-mentioned chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of molecular biology grade.

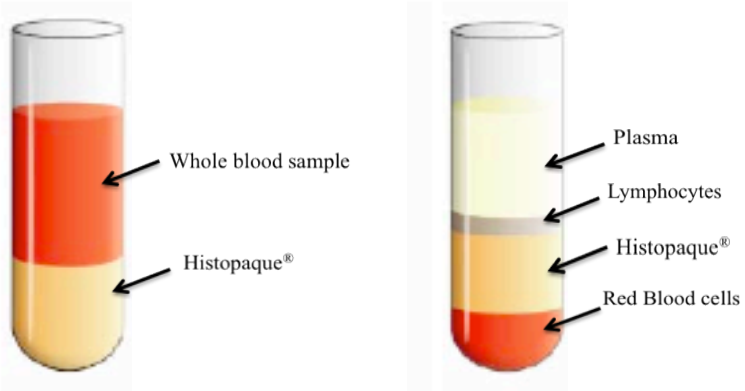


Figure 3.1 Different layers of blood samples before and after separation by gradient centrifugation using Histopaque®

Different versions of the Comet assays used:

Preparation of reagents:

The following preparation methods for of buffers and solutions were applied in all the different versions of the Comet assay used in this study and followed published protocols and our own established procedures (Wong *et al*., 2005; Collins *et al*., 2008; Han *et al*., 2011).

Lysis solution:

0.1M disodium ethylenediaminetetraacetic acid (Na_2EDTA; 32.22g/l), 2.5M sodium chloride (NaCl; 146.1g/l), and 10mM Tris (1.211g/l) were dissolved in MilliQ water, with each chemical added individually to ensure complete dissolving, and the final volume made up to 1 litre. Solid sodium hydroxide pellets (NaOH) were added to the solution to adjust the pH to pH10 and stored at 4^{o}C until use. Four hundred microlitres Triton X-100 were mixed with 40ml of the lysis solution (in an approximate portion of 1:100) immediately before use. Na_2EDTA, NaCl, concentrated NaOH pellets and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, US). Tris was purchased from GE Healthcare (Uppsala, Sweden).

10X Electrophoresis buffer (stock):

3M NaOH (120g/l) and 10mM Na_2EDTA (3.72g/l) were dissolved in MilliQ water and the solution was made up to a final volume of 1 litre with MilliQ water. The solution was stored at 4^{o}C. The buffer was diluted 1/10 (1X) with pre-cooled distilled water for use.

Phosphate buffered saline (PBS):

Commercial PBS powder with no calcium and magnesium was dissolved in one litre MilliQ water.

1% w/v standard agarose solution (for coating slides):

Five grams of biotechnology grade standard agarose were dissolved in 50ml of hot distilled water (heated using a microwave oven). Coated slides were prepared in batches

and in advance of experiments. Standard agarose was purchased from Ameresco (Solon, OH, USA).

1% w/v standard agarose in PBS (acting as an anchor for upper LMP agarose gels):

Thirty milligrams of the same standard agarose were dissolved in 3ml PBS with the use of microwave oven.

1% Low melting point (LMP) agarose in PBS (Embedding cells):

Thirty milligrams molecular biology grade LMP agarose were dissolved in 3ml PBS with the use of a microwave oven. LMP agarose was obtained from Sigma-Aldrich (St. Louis, US).

10X Enzyme reaction buffer:

0.4M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 9.953g/100ml), 1M potassium chloride (KCl; 7.46g/100ml), 5mM Na_2EDTA (0.186g/100ml), 0.1M Tris (1.21g/100ml), and 2mg/ml Bovine Serum Albumin (BSA; 0.2g/100ml) were used to make up to 100ml of this solution, using distilled water. Concentrated potassium hydroxide (KOH) was used to adjust the buffer to pH8. The solution was then aliquoted into 15ml portions each in 50ml sterile centrifuge tubes. Aliquots were stored at -80^{o}C, thawed and diluted 1/10 with distilled water (i.e. 1X Enzyme reaction buffer) as required. HEPES, KCl, BSA and KOH were obtained from Sigma-Aldrich at molecular biology grade, except for HEPES which was biotechnology grade.

Working Formamidopyrimidine DNA glycosylase (Fpg) solution:

Three hundred microlitres of enzyme reaction buffer were added to 10µl stock Fpg solution which was stored at -80^{o}C. The working enzyme solution was freshly prepared and kept on ice before use. Stock Fpg enzyme was kindly provided by Professor Andrew Collins (University of Oslo, Norway).

Ro19-8022 photosensitiser:

21mg of Ro19-8022 photosensitiser was dissolved in 50ml 70% ethanol to give a 1mM stock solution. Ro19-8022 was then aliquoted in 1ml portions in 1.5ml micro-centrifuge tubes and stored at -20^{o}C. This was diluted to 1µM with cold PBS for use. Ro19-8022 was a kind gift from Hoffman la Roche and HPLC grade ethanol was obtained from Sigma-Aldrich.

20µg/ml aqueous ethidium bromide:

25mg/ml stock ethidium bromide (stored at room temperature in the dark) was diluted 1/10 with distilled water. Ethidium bromide was obtained from Sigma-Aldrich at biotechnology grade.

Standard alkaline comet assay:

Clean microscope slides were pre-coated with 1%w/v standard agarose solution (dissolved in distilled water) to ensure a firm anchorage of the upper layers of agarose gels. Seventy microlitres of 1%w/v standard agarose (dissolved in PBS) were added onto two separate areas of a pre-coated slide and each gel area was covered with a cover slip

(18mm x 18mm) until it was set. This gave two agarose gels on one single microscope slide. After washing the thawed cryopreserved cells with PBS twice, 1.5ml 1%w/v low melting point agarose (dissolved in PBS, at 37^{o}C) was applied to re-suspend the washed cell pellet, and 70µl of the cells-LMP agarose mixture was layered on top of the 1%w/v standard agarose gels. The gels were covered with cover slips and left, until it had solidified.

The slides (with cover slips removed) were then placed in approximately 40ml lysis solution (with 0.4ml Triton X-100 added just before incubation) in a Coplin jar at 4^{o}C for 60 minutes in order to disintegrate the cell membranes and nuclear envelop and to degrade histones. After this, the slides were incubated twice with 1X Electrophoresis buffer (preparation as mentioned in previous section) in a Coplin jar at 4^{o}C for 20 minutes for DNA unwinding. Slides were then placed on the platform of the electrophoresis tank (Bio-Rad Laboratories, Hercules, CA, USA) filled with cold 1X Electrophoresis buffer. The tank was put in a tray and surrounded with ice to keep the electrophoresis process cool. Electrophoresis was run for 30 minutes at a constant 25V and the current was set to 300mA by adjusting the buffer volume. During electrophoresis the relaxed DNA loops (containing single-strand breaks) migrated slightly towards the anode, to form a comet 'tail' within the gel. After electrophoresis, the slides were neutralised with neutralising buffer (3 times at 4^{o}C for 5 minutes) in a Coplin jar to minimise the formation of crystals in the gels. After neutralisation, the slides were left to air dry at room temperature for later scoring.

Enzyme (Fpg)-assisted comet assay:

Formamidopyrimidine DNA glycosylase (Fpg) creates strand breaks at DNA sites containing oxidised purines, i.e. 8-oxoGua (generally known as "Fpg-labile sites"). This version of the comet assay is a modification of the standard alkaline comet assay described above, and was performed as follows.

After the lysis step, the slides were transferred to another Coplin jar and further incubated with enzyme reaction buffer at 4^{o}C (3 times, 5 minutes each) to remove the remaining lysis solution and equilibrate gel conditions. Slides were removed from the Coplin jar and drained. Fifty microlitres of the working Fpg solution was added directly onto the gels which were then covered loosely with Parafilm^{TM}; 50µl of the enzyme reaction buffer alone (no Fpg) was added onto control gels. The slides were then placed flat inside a moisture chamber and incubated at 37^{o}C in 5% CO_2 for 30 minutes. Treatment (Fpg) and control (buffer only) slides were placed in different chambers in order to avoid any cross-contamination. Parafilm^{TM} covers were then removed and the slides were further processed through the DNA unwinding, electrophoresis and neutralisation steps following the same procedures as in the standard alkaline comet assay.

Cell extract version of the comet assay:

The procedure of this version of the comet assay is similar in many ways to the Fpg-assisted comet assay as described in the previous section, however, the cells run through it are 'substrate cells' (in this case HeLa cells) that have been purposefully damaged, in a controlled manner, and the damage is revealed by the activity of the DNA repair enzyme

hOGG1 present in extracts of lymphocytes from our volunteers. This version of the comet assay is designed to assess the activity of the hOGG1 (Collins *et al*., 2003). In this version, it is the 'substrate cells (the damaged HeLa cells with oxidation-induced lesions) that are embedded in agarose. Since hOGG1 in the lymphocyte extract will convert the oxidation-induced lesions in the DNA of the HeLa cells to strand breaks (in the same way that Fpg induces lesions), the higher the enzyme activity, the greater the level of damage that will be revealed in the damaged HeLa (as %DNA in tail).

Culture of HeLa cells:

An aliquot of stock HeLa cells (from ATCC (Virginia, USA) was removed from liquid nitrogen and thawed in a 37^{o}C water bath. The HeLa cells were then transferred to a T75 culture flask with 15ml RPMI-1640 supplemented with 10% FBS (complete medium). The cells were incubated overnight in a humidified incubator with 5% CO_2 at 37^{o}C. The cells were washed with warm PBS twice. The medium was replaced with fresh complete medium the day after removal from nitrogen (the cells should already be anchored onto the culture flask surface by this time), as the freezing medium may contain preserving chemicals. RPMI-1640 and FBS were certified and obtained from Gibco (Grand Island, NY, US).

HeLa cells were maintained in 100mm culture dishes in a humidified incubator with 5% CO_2 at 37^{o}C. When an 80% confluency in the dish/flask was reached (with the cells growing in a monolayer), the cells were sub-cultured at 3 to 4 day intervals. The cells were rinsed with warm PBS twice, then detached from the dish surface with 500µl 0.25%

trypsin-EDTA. The dish was then incubated at 37^{o}C for 3-5 minutes. A prolonged incubation time must be avoided as the trypsin-EDTA may digest the cells, causing damage. After incubation, 3ml of complete medium was added to inactivate the trypsin-EDTA and the cell suspension was transferred to a clean 15ml centrifuge tube. The cell suspension was centrifuged at 1500rpm for 5 minutes at 4^{o}C. The supernatant was removed and the resulting cell pellet was re-suspended in 1ml of complete medium. One hundred microlitres of the cell suspension were then added to a new 100mm culture dish with 8ml of complete medium and cultured until the next passage. The remaining cell suspension was used for preparation of substrate cells for the comet assay. Trypsin-EDTA was purchased from Caisson Laboratories (North Logan, UT, US).

Preparation of substrate HeLa cells:

The cultured HeLa cells were seeded overnight at a population of 3x10^{6} cells in each 60mm culture dish with 5ml complete medium at 37^{o}C. Several dishes were prepared in one single batch to maintain the homogeneity of cells to be used for the following photosensitisation treatment. The cells were rinsed twice with 2ml PBS and, in preliminary experiments performed to check that HeLa cells were being adequately damaged by the photosensitiser and light, but not by either light or photosensitiser alone, aliquots of cells were subjected to one of the following four different treatments for five minutes:

a. PBS without illumination; b. PBS with illumination; c. 1μM Ro19-8022 without illumination; d. 1μM Ro19-8022 with illumination. The illumination was achieved by a 500W tungsten halogen lamp (Philips Focusline, China) at a distance of 33cm, where the

dish was placed on top of a box filled with ice to avoid heat damage to cells. This controlled procedure (d) created oxidation-induced lesions in the DNA of the HeLa cells. These lesions are recognised by hOGG1, which creates strand breaks at the lesions. The cells were then rinsed with cold PBS twice in the dark, and trypsinised with 500µl 0.25% trypsin-EDTA. The dishes were then incubated at 37^{o}C for 3 minutes followed by addition of 3ml complete medium. The cells that underwent different treatments were then transferred, separately, to clean 15ml centrifuge tubes and centrifuged at 1500rpm for 5 minutes at 4^{o}C. The cells were washed twice with cold PBS, and the cell concentration was determined by using a haemocytometer. The cell pellet was finally re-suspended in cold freezing medium containing 90% FBS and 10% DMSO at a cell density of $3x10^{6}$ cells/ml. Aliquots of 0.3ml cell suspension of HeLa cells treated with photosensitiser and light (treatment (d) above) were put into 1.5ml microcentrifuge tubes. Enough aliquots of the same batch of damaged substrate cells were prepared for the preparation of up to 28 slides for comet assay testing. The aliquots were placed in a polystyrene box, and cryopreserved at -80^{o}C.

In order to minimise inter-treatment variations, cells from different dishes of the same treatment (d) were pooled together before subsequent washing, counting and aliquoting steps. Variation due to separate passages of HeLa cells was avoided by using cells from the same passage in each experiment.

Preparation of cell extract from subjects' lymphocytes

Harvesting of lymphocytes from whole blood using Histopaque®-1077 was as described above. However, instead of adding freezing medium to the cell pellets for cryopreservation, the cell pellets were washed twice with cold PBS, centrifuged and as much PBS as possible removed (to avoid diluting the cell extract) and then flash frozen with liquid nitrogen. The pellets were stored at -80°C. On the day of testing, 65µl of commercial mammalian cell lysis solution (Fermentas, Vilmius, Lithuania) were added to each pellet, and the mixture was then vortexed vigorously for 15 seconds and put on ice for 10 minutes. The cell lysate was then centrifuged at 12,000rpm for 5 minutes at 4°C. Fifty-five microlitres of the supernatant were transferred to another microcentrifuge tube and mixed with 220µl of cold enzyme reaction buffer. This mixture is referred to as the cell extract

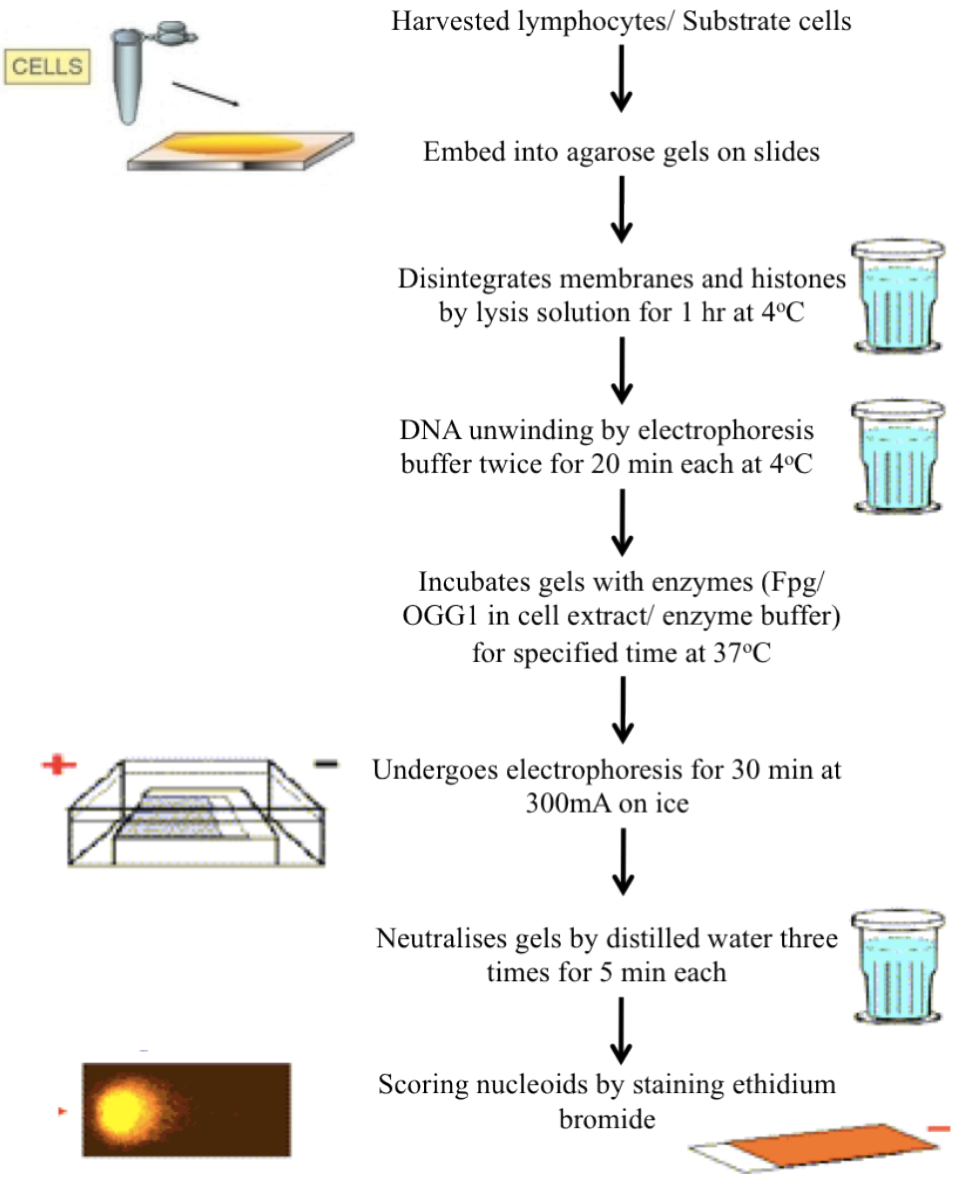
Cell extract version of the comet assay for samples:

An aliquot of damaged substrate cells was thawed and washed with PBS twice. The cells were then embedded with 1% LMP agarose onto microscope slides pre-coated with 1% standard agarose. The slides were put through the lysis step and, in a step analogous to that of the Fpg incubation step described above, 50µl of the cell extract (prepared as described above and containing hOGG1) were then added onto each 'treatment' gel whereas enzyme reaction buffer of the same volume was added onto the control gel. The slides were put into a moisture chamber, and incubated at 37°C for 30 minutes. The subsequent DNA unwinding, electrophoresis and neutralisation steps were performed in the same way as the Fpg-assisted comet assay.

Scoring the comet images:

After air drying the slides, the comet images of the nucleoids were scored. A computer image capture and analysis software system for the comet assay, Komet 5.5 (Kinetic Imaging Limited, Liverpool, Merseyside, UK) was used. Forty microlitres of 20µg/ml aqueous ethidium bromide, a fluorescent DNA stain, were added onto each gel just before scoring, covered with cover slips, and scored under a fluorescent microscope (Nikon Eclipse E600, Nikon, Tokyo, Japan) linked to the computer in the dark room. The percentage DNA content in the comet tail was recorded in each nucleoid scored. A total of 50 nucleoids per gel and 100 nucleoids per treatment were scored. A simplified workflow of the Comet assay is presented in Figure 3.2.

Figure 3.2 A simplified diagram showing the flow of the Comet assay



Gene expression study:

Preparation of buffers:

Red blood cell (RBC) lysis solution:

A 155mM ammonium chloride (NH_4Cl; 8.29g/l), 10mM potassium bicarbonate ($KHCO_3$; 1.0012g/l), and 1mM EDTA solution was prepared in distilled water. The solution was autoclaved and stored at room temperature. NH_4Cl and $KHCO_3$ were obtained from BDH Laboratory Supplies (Poole, England**).**

20X Borax buffer:

200mM NaOH (8g/l) and 725mM boric acid (H_3BO_3; 45g/l) solution was prepared in distilled water, and the solution was stored at room temperature and diluted with distilled water to 1X (i.e. diluted 1/20) before use with distilled water. H_3BO_3 was obtained from GE Healthcare (Uppsala, Sweden).

Total RNA extraction from blood samples

Total RNA extraction was performed using the commercial kit, Roche High Pure RNA Extraction (Roche Diagnostics, Almere, The Netherlands) and following the manufacturer's instructions. In brief, 3ml of red blood cell lysis solution were added to 1.5ml blood. The mixture was placed on a rocker at room temperature for 10 minutes. The mixture was centrifuged at 1500rpm for 5 minutes at 4^oC after complete lysis of red blood cells. The supernatant was removed and the pellet was re-suspended with 1ml RBC lysis solution. The suspension was then centrifuged at 1500rpm for 3 minutes. The supernatant was discarded, removing the remaining red blood cells around the pellet as

much as possible. Four hundred microlitres of lysis buffer were added to the pellet and the mixture was then vortexed for 15 seconds. The lysate was loaded onto the membrane of the column provided in the kit according to the manufacturer's protocol. The column was centrifuged at 9500rpm for 15 seconds at room temperature. The flow-through, which included the cell debris resulting after lysis, was discarded. To prepare the DNase I enzyme solution, 10µl DNase I was mixed with 90µl incubation buffer for each sample. One hundred microlitres of the DNase I enzyme solution were loaded onto the membrane where RNA was bound to the silica component. The column was then incubated at room temperature for 15 minutes. After incubation, 500µl wash buffer I were added onto the membrane and the column was spun at 9500rpm for 15 seconds. The washing step was repeated again with 500µl wash buffer II. A final washing step was performed with 200µl wash buffer II and the column was centrifuged at 12000rpm for 2 minutes. The upper reservoir of the column with membrane was transferred to a new collection tube. Forty microlitres of elution buffer were added onto the membrane, and the RNA sample was eluted to the collection tube after spinning at 9500rpm for 1 minute. The RNA concentration of each sample was measured using NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, US) and recorded. Reverse transcription was performed immediately after RNA extraction.

Reverse transcription:

Reverse transcription was performed using the commercial kit, Maxima® First Strand cDNA Synthesis Kit for reverse transcription quantitative PCR (RT-qPCR; Thermo Scientific, Runcorn, UK) and according to the manufacturer's protocol. All reagents for

the reverse transcription were thawed from -20°C. The reagents were vortexed gently and put on ice before use. For each reaction in a 0.2ml PCR microcentrifuge tube, 1µl of the oligo$(dT)_{18}$ primers was added with 11µl RNA from each sample. Since the yield of RNA extraction and the amount of blood collected from volunteers were consistent, a maximum of 11µl neat RNA sample were used in each reaction, three reactions were made from each of the 40µl sample. The mixture was then spun down and incubated in the thermocycler (GeneAmp® PCR system 9700, Applied Biosystems, Foster City, CA, USA) at 70°C for 5 minutes to reduce RNA secondary structures. The samples were then put on ice immediately after incubation. During the five-minute incubation, a master mix for reverse transcription was prepared. For each reaction, 4µl 5X Reaction buffer (final concentration: 1X) was mixed with 1µl RNase inhibitor, 2µl 10mM dNTP (final concentration: 1mM) and 1µl reverse transcriptase extracted from Moloney Murine Leukaemia Virus (MMLV; final concentration: 20U/µl). After preparation of the master mix, 8µl of the master mix were added to the RNA-primer mixture, making up a total of 20µl of reaction mixture. The reaction mixture was vortexed briefly and spun down. The reverse transcription was then activated at 37°C for 5 minutes, followed by the initiation of the reaction at 42°C for 1 hour. The reaction was stopped by placing the tubes at 70°C for 10 minutes to inhibit the enzymatic reaction. The cDNA samples were then stored at -80°C until later analysis.

Real-time PCR:

Neat cDNA samples were thawed, mixed and put on ice before use. The samples were then diluted into 1/10 with nuclease-free water, and 1µl of the diluted sample was added

into each well of PCR strips according to the work list/sample map prepared for each experiment. The samples were put on ice until the master mix was prepared. The master mix contained several components, 10X Reaction buffer (final concentration: 1X), 2mM dNTP (final concentration: 0.2mM), 25mM $MgCl_2$ (final concentration varied according to genes to be amplified), 5U/µl AmpliTaq® Gold Polymerase (1.2U/reaction; used in the *in vitro* study) or 5U/µl HotStarTaq® DNA Polymerase (0.4U/reaction; used in the *in vivo* study), 10µM forward and reverse primers of a particular gene (final concentration: 0.3µM; information of primers is provided in Table 3.1, and the primers used in this study were set using Oligo 6 and run through BLAST to ensure they were specific for the area being amplified), 100X SYBR® Green (final concentrations in the *in vitro* and *in vivo* studies are specified in later sections; from Applied Biosystems, Foster City, CA, US), 25µM ROX® (final concentrations are specified in later sections; from Applied Biosystems, Foster City, CA, US), and nuclease-free water (Gibco, Grand Island, NY, US). Unless specified, reagents, i.e. 10X Reaction buffer, dNTP, $MgCl_2$, and *Taq* polymerase, used in the *in vitro* study were obtained from Applied Biosystems (Foster City, CA, US) and those used in the *in vivo* study were from Qiagen (Hilden, Germany). Different sources of reagents were applied due to limited resources to seek for a more economic option.

Once the master mix was prepared, it was thoroughly vortexed, and then 9µl of the master mix were added to each well containing the diluted cDNA samples. The final volume of the total reaction mixture including cDNA sample was 10µl. The reaction mixtures were then spun for several seconds. Air bubbles were removed before starting

the reaction as were any droplets that may have adhered to the lid of the reaction tube as these may affect the reading of the detector of the real- time PCR instrument (ABI 7500 Real-Time PCR System from Applied Biosystems, Foster City, CA, US). To start the reaction, the reaction mixture was incubated at 95^{o}C to activate the *Taq* polymerase enzyme for 10 minutes followed by 35-40 cycles, with each cycle consisting of a denaturation (strand separation) stage at 95^{o}C for 0.5-1 minute, an annealing stage at 52^{o}C for 0.5-1 minute and an extension stage at 72^{o}C for 0.5-1 minute. The reaction was terminated with a final extension at 72^{o}C for 10 minutes. For each run, a dissociation curve measurement was performed to ensure the purity of the amplified products. Performing dissociation curve analysis after PCR, fluorescent signals are obtained by gradually ramping the temperature of reaction solutions from 60 to 95 °C. The increase in temperature causes PCR products to undergo denaturation, i.e. strand separation, and SYBR® Green can no longer bind to the double stranded product, this is accompanied by a drop in fluorescence of the reaction mixture. The cycle number of amplification threshold (Ct) of each sample was recorded. Specific primers and cycling conditions are further specified in the corresponding chapters.

To further verify the identity of the amplified products, each PCR product was resolved in a 2% agarose gel. The bands, representing the product size, were visualised under UV illumination. A more definitive confirmation of the amplified product was to perform DNA sequencing. This was performed on ABI PRISM®310 Genetic Analyzer (Applied Biosystems, Foster City, CA, US) to confirm the authenticity of the amplified products. The verification of amplified products was done during optimisation of suitable primers.

Gel electrophoresis:

Setting agarose gel:

The agarose gel was prepared in 2%w/v in 1X borax buffer. Agarose powder was weighed and poured into a conical flask. The corresponding volume of 1X borax buffer depending on the gel size was used to dissolve the powder to give 1.5% of agarose. The weight of the agarose solution and conical flask was recorded and an extra 30g of distilled water was added to make up any water which may have been lost due to evaporation and to maintain the buffer concentration. The agarose solution was heated in a microwave oven at high power for 5 minutes. The agarose solution was weighed again and distilled water was added to resume the original weight. The agarose solution was mixed thoroughly and poured into a gel solidification set-up. Bubbles were removed using the aid of disposable pipette tips. The gel was allowed to set for 30 minutes and covered with aluminium foil. Once the gel was set, it was used directly in the electrophoresis tank for sample loading or it was stored in a foil-covered box filled with 1X Borax buffer at 4^{o}C until required.

Sample loading:

Before loading PCR products, a piece of Parafilm^{TM} was prepared, and one 2µl drop of loading dye was added onto the Parafilm^{TM} for each sample. Five microlitres of PCR product was mixed with the loading dye and the mixture was transferred to a well of the gel. One microlitre of SYBR$^{®}$Safe (Applied Biosystems, Foster City, CA, USA) was mixed with 4µl working GeneRuler (stock solution was diluted with distilled water in a

ratio of 1:9 and stored at 4^{o}C) (Fermentas, Vilnius, Lithuania) before mixing with loading dye and loading onto the gel. Electrophoresis was then performed in 1X borax buffer at 100V for 60 minutes covered with aluminum foil.

Visualisation of PCR products using the Gel Documentation System (GENESNAP):

The platform of the instrument was covered with a piece of cling film and the gel was placed on the platform. The live gel image was then captured by the GeneSnap imaging software (Syngene, Frederick, MD, USA) with ordinary white light in order to adjust the location of the captured image of the gel. The gel was then exposed under UV illumination and the fluorescent signal from the SYBR® Green was observed and captured by adjusting the brightness and contrast of the image taken.

Table 3.1. Information of primers for the genes of interest

Gene	Orientation	Sequence	Size (bp)
CD3ε	Forward	5′-GGCAGGCAAAGGGGACA-3′	236
	Reverse	5′-ACCATGAGGCTGAGGAACGAT-3′	
CD8β	Forward	5′- GACAGTCACCACGAGTTCCT-3′	133
	Reverse	5′- GCTTCACGCTTGTGAGATTGAGAA-3′	
HMOX1	Forward	5′-CAGGCAGAGAATGCTGAGTTC-3′	146
	Reverse	5′-GATGTTGAGCAGGAACGCAGT-3′	
NRF2	Forward	5′-AGTGGATCTGCCAACTACTC-3′	106
	Reverse	5′-CATCTACAAACGGGAATGTCTG-3′	
KEAP1	Forward	5'-ATGCCTCAGTGTTAAAATGACAT-3'	224
	Reverse	5'-CAGGTATCCAAGAATAAATCACA-3'	
BACH1	Forward	5′-TGCGATGTCACCATCTTTGT-3′	100
	Reverse	5′-CCTGGCCTACGATTCTTGAG-3′	
hOGG1	Forward	5′-ACACTGGAGTGGTGTACTAGCG-3′	301
	Reverse	5′-GCCGATGTTGTTGTTGGAGG-3′	
XRCC5	Forward	5′-GCGACAGGTGTTTGCTGAGA-3′	78
	Reverse	5′-GAAAGGGGATTGTCAGTGCCAT-3′	

NQO1	Forward	5′- GAGGTACAGGATGAGGAGAAAAA-3′	84
	Reverse	5′- CTGGTTGTCAGTTGGGATGGA-3′	
GSTα	Forward	5′- GGCTGACATTCATCTGGTGG-3′	199
	Reverse	5′- CCTGAAAATCTTCCTTGCTTCTTCT-3′	

Western Blotting:

Preparation of buffers:

10% w/v Sodium Dodecyl Sulphate (SDS):

5g SDS were dissolved in 50ml distilled water and stored at room temperature. SDS was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

1.5M Tris-HCl (pH8.8):

36.3g Tris were dissolved in 190ml distilled water. The solution was then adjusted to pH8.8 with concentrated hydrochloric acid. The solution was eventually made up to 200ml with distilled water and stored at 4^{o}C.

0.5M Tris-HCl (pH6.8):

12.1g Tris were dissolved in 190ml distilled water. The solution was then adjusted to pH6.8 with concentrated hydrochloric acid and made up to 200ml with distilled water and stored at 4^{o}C.

10% Ammonium Persulphate (APS):

1g APS was dissolved in 10ml distilled water. The solution was aliquoted into 1.5ml eppendorfs and stored at -20^{o}C. APS was obtained from Sigma-Aldrich at molecular biology grade (St. Louis, MO, USA)

10X Running buffer (stock):

30.3g Tris, 144g glcyine and 10g SDS were dissolved in distilled water to a final volume of one litre and this was stored at room temperature. 1X working running buffer was prepared by diluting 100ml of 10X running buffer with 900ml of distilled water, and stored at room temperature. Glycine was obtained from GE Healthcare (Uppsala, Sweden).

10X Transfer buffer (stock):

30.3g Tris and 144g glycine were dissolved in 1 litre of distilled water and stored at 4^{o}C.

1X Transfer buffer (working):

100ml 10X transfer buffer was diluted with 700ml distilled water and 200ml methanol. The working buffer was stored at 4^{o}C. Methanol was obtained from Sigma-Aldrich at molecular biology grade (St. Louis, MO, USA).

10X TBS (stock; pH7.6):

24.2g Tris and 80g sodium chloride were dissolved in 900ml distilled water. The solution was then adjusted to pH7.6 with concentrated hydrochloric acid. The solution was eventually made up to 1 litre with distilled water and stored at 4^{o}C. 1X working TBS was prepared by diluting 100ml of 10X TBS with 900ml of distilled water, and stored at room temperature.

TBS-Tween (0.1% w/v Tween 20):

1 litre of 1X working TBS at pH7.6 was mixed with 1ml Tween 20, and stored at room temperature. Tween 20 was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Stripping buffer:

7.58g Tris and 20g SDS (20g/l) were dissolved in 1 litre of distilled water. β-mercaptoethanol was added in a concentration of 7µl/ml stripping buffer just before use and was obtained from Sigma-Aldrich (St. Louis, MO, USA).

6X Loading Dye:

1.2g SDS, 6mg bromophenol blue, 4.7ml glycerol and 1.2ml Tris (pH6.8) were dissolved in 2.1ml distilled water. β-mercaptoethanol was added in a dilution of 1 in 20 just before use.

10% Resolving gel:

3.8ml distilled water were mixed with 2ml of 1.5M Tris (pH8.8), 80µl of 10% SDS, 2ml of 40% Acrylamide-Bis, and 77µl of 10% APS. 13µl TEMED were added and mixed well just before setting the gel in the glass plates. Acrylamide-Bis at a concentration of 40% was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

4% Stacking gel:

3.15ml distilled water were mixed with 1.25ml of 0.5M Tris (pH6.8), 50µl of 10% SDS, 500µl of 40% Acrylamide-Bis, and 38µl of 10% APS. 12µl TEMED were added and mixed well just before setting the gel in the glass plates.

Preparation of a gel:

An assembled gel sandwich (the clip with glass plates, Bio-Rad Laboratories, Hercules, CA, USA) was prepared and placed on the holding stand. A comb was placed in between the glass plates and a line was marked 1cm beneath the teeth of the comb where the level of resolving gel should reach. The comb was then removed. The resolving gel solution was prepared according to the recipes as given above. The percentage of the gel used was dependent upon the molecular weight of the particular products of interest. Bubbles should be avoided during the preparation of the gel. The prepared resolving gel solution was immediately transferred to the gel chamber enclosed with glass plates until it reached the mark previously drawn. The gel solution was overlaid with MilliQ water until the chamber was fully filled in order to pack the resolving gel, and the gel was allowed to set for 30 minutes at room temperature. After the gel was set (two phases were seen in the chamber), MilliQ water on top of the gel was discarded and the leftover moisture was removed with filter paper with care so as not to touch the gel. Stacking gel solution was prepared according to recipes given above, and transferred directly onto the resolving gel until the chamber between glass plates was filled up. The casting comb was then inserted into the stacking gel solution at a slant to avoid introduction of bubbles between the wells and beneath the teeth of the comb. The stacking gel solution was allowed to set for 1.5 hour at

room temperature. The gel was either used immediately or stored in 1X Running buffer at 4^{o}C.

Sample preparation:

Fast frozen cell pellets collected from whole blood (sample collection as described in Chapter 5) were thawed and centrifuged at 12000rpm for 15 minutes. The remaining, thawed PBS was discarded to avoid dilution of protein. Twenty microlitres of commercial mammalian cell lysis reagent were added to each sample, and the mixture was vortexed vigorously for 30 minutes with intermittent cooling on ice. The mixture was centrifuged at 12,000rpm for 30 minutes at 4^{o}C. The resulting supernatant was then transferred to a labelled 0.2ml PCR microcentrifuge tube. Protein concentrations of each sample were then measured and recorded using NanoDrop for later dilution use. NanoDrop was used to measure the protein concentration of each sample by reading the absorbance due to the limited amount of protein sample collected. Compared to conventional Bradford's assay for measuring protein concentrations, the current method requires only 2μl of the sample instead of 200μl. The samples were stored at -80^{o}C until use. Upon testing, the protein samples were diluted with cell lysis reagent to give a total of 10μg protein in the aliquot. The diluted protein aliquot was then made up to 20μl with loading dye (with β-mercaptoethanol added in a dilution of 1 in 10 in advance) in 0.2ml PCR microcentrifuge tube. The protein mixture was then denatured in a thermal cycler at 95^{o}C for 5 minutes, and loaded into the wells of the gel.

Loading of samples and running gels:

Glass plates were clipped onto the running casket where the shorter glass plate faced inwards. The casket was placed in the tank. The centre of the tank enclosed by two sets of glass plates was filled with 1X running buffer slowly avoiding introduction of bubbles into the wells. Ten microlitres of each denatured sample was loaded into the wells. For each gel, a protein ladder as a size reference was also loaded into one of the wells at either end of the gel. After loading the samples into the wells, the tank surrounding the glass plates was filled with 1X running buffer to half full as indicated on the tank. The gel was allowed to run after connecting to power (PowerPac, Bio-Rad, Hercules, CA, USA) at room temperature at a constant voltage of 100 volts for 120 minutes or according to the position of the loading dye. The set-up was dismantled after running for subsequent transfer of proteins in the gel onto a membrane.

Preparation of transfer of proteins onto membrane:

Buffers were pre-cooled at 4^{o}C. The polyvinylidene fluoride (PVDF) membrane of 0.2µm (Merck Millipore, Bedford, MA, USA) was first activated in methanol at room temperature for 5 minutes with rocking. The membrane was then washed with distilled water with rocking at room temperature for 5 minutes, three times. A corner of the membrane was cut in order to distinguish the side of the membrane with proteins transferred onto it from the other side. Transfer buffer was poured into a tray with the transfer sandwich, two sponge pads and 4 pieces of filter paper pre-wet with transfer buffer.

Transfer of proteins from gel to membrane:

The glass plates were separated with a spatula, leaving an intact gel. Once the shorter plate was removed, the stacking gel was removed with care. Resolving gel was then carefully separated from the spacer plate and transferred to the tray with transfer buffer. A complete transfer sandwich was set up in the order of negative side of cassette (black), sponge pad, two pieces of filter paper, gel, membrane, two pieces of filter papers, sponge pad, and positive side of cassette (white). A thick glass rod may be used to roll the sandwich to remove any air bubbles trapped between the gel and the membrane. The cassette was closed and locked immediately. The transfer cassette was put into a trans-blot module where the negative side of the cassettes was with the negative side of the trans-blot module. The tank was placed into a bucket filled with ice, and an ice pack was put in the free space in the trans-blot module. The tank was then filled up with the cold transfer buffer. During the transfer step, the buffers and equipment become very hot. In order to maintain a cool system, the whole set up was placed onto a stirrer and a magnetic lead can be placed into the tank and the lid of the tank can be covered up with ice during transfer. The transfer step was performed after connection to the power at a constant voltage of 100 volts for 120-150 minutes.

Confirmation of transfer of proteins:

In order to confirm the proteins were transferred from the gel to the membrane, the membrane was incubated with *Ponceau S* dye for 20 seconds, and protein bands were seen on the membrane provided there was adequate transfer of the proteins. The membrane was then washed with distilled water for 1 hour with rocking.

Trimming of PVDF membranes:

To save resources, membranes were cut and probed against different primary antibodies according to the sizes of different proteins of interest. Cutting of membrane has to be done very carefully and it is not suggested to stain two proteins with similar molecular weight. *Ponceau S* can be used to establish the best position to cut the membrane.

Blocking of PVDF membranes:

Since PVDF membranes have high affinity for proteins, the sites where there is no protein transfer must be blocked with BSA so that these areas will not bind non-specifically to primary antibodies. Five percent BSA was prepared in cold TBST and used as a blocking solution. After the transfer step, the membrane was placed into a plastic box with 5% BSA in TBST, and incubated for one hour with rocking at room temperature.

Incubation of membrane with primary antibody:

After blocking, 2% BSA was prepared in cold TBST. Primary antibody was diluted with 2% BSA in TBST and mixed well. The dilution of each primary antibody varied depending on the protein of interest. HO-1 was obtained from Abcam (Cambridge, MA, USA), while CD3ε was from Cell Signaling (Beverley, MA, USA). In this project, the dilutions of each primary antibody were HO-1, 1:500 and CD3ε, 1:200. The blocking reagent was removed as much as possible and diluted primary antibody in 2% BSA was added to the membrane. The membrane was then incubated overnight at 4^{o}C with

continuous rocking. The used, diluted primary antibody after incubation was kept at 4^{o}C until next use.

Incubation of membrane with secondary antibody:

The membrane was placed into another plastic box and washed with TBST 3 times for 5 minutes each. To prepare secondary antibody solution, 2% BSA in cold TBST was prepared, and the secondary antibody was diluted in a ratio of 1:2000. It is of importance to note that the choice of secondary antibody is based on the animal in which the primary antibody is raised, i.e. if the primary antibody is raised in mice, the secondary antibody must be anti-mouse. After discarding all the washing TBST, diluted secondary antibody with 2% BSA was added to the membrane for 1-hour incubation at room temperature followed by a washing step of three times with TBST for 5 minutes each. The membrane was left in minimal volume of TBST until the chemiluminescent solution was prepared.

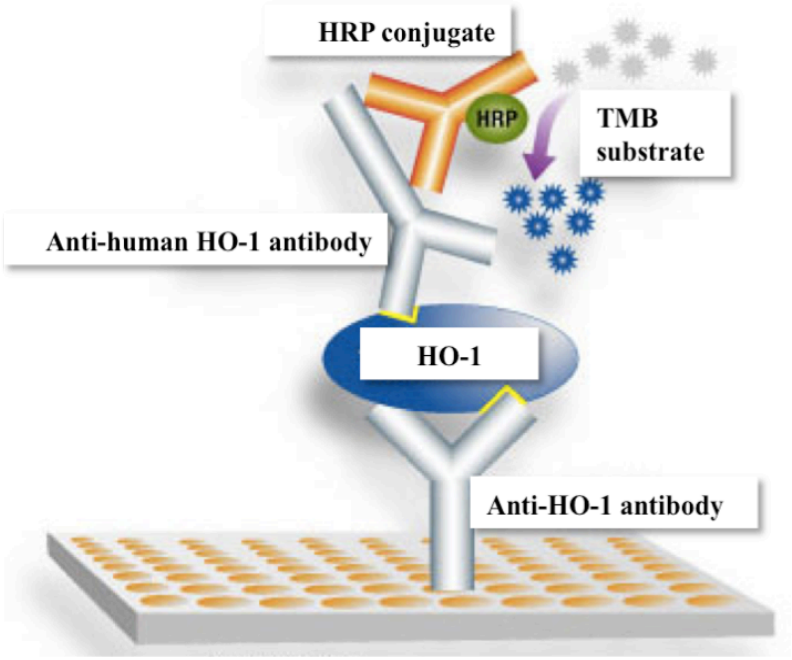
Detection of proteins of interest:

Enhanced chemiluminescent (ECL) substrate reagent (Immobilon ECL, Millipore, Billerica, MA, USA) was purchased ready to use. The membrane was incubated in the prepared ECL substrate reagent for 2 minutes with constant pipetting of reagent onto the membrane. The membrane was then placed on the platform of the image analysis instrument (Kodak Image Station 4000MM, Kodak, Rochester, NY, USA).

Determination of plasma HO-1 concentrations:

Plasma HO-1 concentrations were determined using enzyme-linked immunosorbent assay (ELISA) (Enzo Life Sciences, Farmingdale, NY, USA). From the concentration range reported from other human studies, no dilution of plasma samples is needed to measure plasma HO-1 concentration in healthy human subjects using this particular kit (Bao *et al*., 2010; Bao *et al*., 2012; Walther *et al*., 2012). According to the manufacturer's protocol, 100µl of heparinised plasma sample or standard solutions were added to wells of the immunoassay plate pre-coated with anti-HO-1 antibody to capture the HO-1 protein. The plate was then incubated in the dark at room temperature for 30 minutes. Each well was washed thoroughly with 1X wash buffer six times, and 100µl diluted anti-human HO-1 antibody were added to each well and incubated in the dark at room temperature for 1 hour. The wells were washed again with 1X wash buffer six times. Another 100µl of diluted horseradish peroxidase (HRP) conjugate were added to the wells, and incubated further in the dark at room temperature for 30 minutes, followed by washing with 1X wash buffer six times. After washing, 100µl tetramethylbenzidin (TMB) substrate were added to each well and incubated in the dark at room temperature for 15 minutes. Another 100µl of stop solution were added to each well, and the absorbance at the wavelength of 450nm of each well was measured using a spectrophotometer. A simplified diagram is shown in Figure 3.3 of the basic principle of this immunoassay. The plasma HO-1 concentration of each sample was obtained from the plot of the HO-1 standard curve.

Figure 3.3 Basic principle of immunoassay (adapted from www.microscopeblog.com)



Total antioxidant capacity of human plasma samples:

This was performed following our established procedures for the FRAP assay (Benzie & Strain, 1996).

Preparation of buffers:

All reagents, except 300mM acetate buffer, were kept at 4^{o}C until use, and used within two weeks. Acetate buffer can be stored at room temperature for up to 6 months.

300mM Acetate buffer:

3.1g sodium acetate trihydrate (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 600ml distilled water and 16ml glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA) were added. The solution was made up to a litre with distilled water and pH was adjusted to pH3.6 with glacial acetic acid.

Iron (III) chloride solution:

270mg iron(III) chloride ($FeCl_3 \bullet 6H_2O$, Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 50ml MilliQ water.

TPTZ solution:

78mg 2,4,6-tripyridyl-s-triazine (TPTZ, Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 25ml 36mM hydrochloride solution. The solution was discarded if colour change was observed.

1000µM Iron (II) Calibrator:

27.8mg iron(II) sulphate ($FeCl_2 \bullet 7H_2O$, Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 100ml MilliQ water using a volumetric flask. The solution was discarded if any precipitate was observed.

Working FRAP reagent:

Working reagent was prepared from acetate buffer, $FeCl_3$, and TPTZ solution in a ratio of 10:1:1. The working reagent was discarded if a blue colour was observed.

Measurement of plasma total antioxidant capacity (as the FRAP value):

The measurement of plasma total antioxidant capacity applied in this study is based on the ability of samples to reduce Fe(III) to Fe(II). In working reagent for the assay, ferric-tripyridyltriazine (Fe(III)/TPTZ) complex is formed in excess. This complex can be reduced by antioxidants at low pH, i.e. pH 3.6, provided by the acetate buffer, and an intensive blue colour will develop. This change in colour can be measured by examining the change in absorbance at a wavelength of 593nm. Established protocol from our group was adopted in this study (Benzie & Strain, 1996). A centrifugal analyser, the Cobas FARA (Roche Diagnostics, Indianapolis, US) was used to measure the total antioxidant capacity of plasma samples. Heparinised plasma samples were used in this test and 100µl of the sample was added to the designated FARA sampling cups. The sampling cups were then loaded onto the Cobas FARA analyser. The change in absorbance of samples at a wavelength of 593nm was recorded, i.e. FRAP. The calculation of FRAP is as follows:

FRAP value (in µM) = [(ΔA_{593} of test sample from 0-4 minutes)/ (ΔA_{593} of calibrator from 0-4 minutes)] X 1000µM $FeCl_2$ calibrator

Biomarkers of Interest in Human Plasma Samples:

Various biomarkers were examined using heparinised plasma. These included the FRAP value, total bilirubin, cholesterol (CHL), triglycerides (TG), high-density lipoprotein cholesterol (HDL), uric acid, and high-sensitivity C-reactive protein (hsCRP). Except for the light sensitive and unstable total bilirubin and the FRAP value, which were performed on fresh samples, measurement of all the other biomarkers were performed in batches on stored (at -80^{o}C) samples. Examination of all these biomarkers was performed using a Hitachi Model 902 Automatic Analyser (Roche Diagnostics, Indianapolis, USA), and the reagents for each biomarker were purchased from Roche Diagnostics **(**Indianapolis, USA**)**.

Plasma polyphenols were measured (by another member of our group, and results provided to this investigator) using LC-MS/MS. In brief, aliquots of thawed plasma samples were stabilised with aqueous ascorbic acid containing EDTA.Na_2.$2H_2O$, followed by enzyme pre-treatment (for measurement of total (conjugated plus free) catechin measurement) by adding β-glucuronidase and sulphatase. Internal standard of taxofolin was added to the mixture and incubated at 37^{o}C for 45 minutes. Chilled methanol was added to extract catechins and precipitate protein from samples after samples had been cooling on ice water for 1 minute. The methanol fraction containing catechins was collected after centrifugation and mixed with aqueous ammonium acetate (pH 4.5) before HPLC-MS/MS analysis. Aqueous Na_2HPO_4 was added for samples without enzyme treatment (free catechins measurement) and the incubation at 37^{o}C for 45 minutes was skipped.

Free and total EGCG, EGC, ECG, EC and C of the test samples and of a 1.5%w/v infusion of green tea were measured using an HPLC system (Agilent 1200 Series, quaternary pump, G1311A and micro vacuum degasser, G1322A). All data were acquired and manually processed by the Analyst® Software 1.4.2. Using this HPLC-MS/MS method, limits of quantitation (LOQs, in nmol/l) of: EGCG 3.6; EGC 7.3; EC 58.3; ECG 1.8; C 58.3, were found. Catechin concentrations were calculated by corresponding peak area ratios (standard/internal standard) compared with the external standard and using the calibration curves generated by injecting 0.78 to 25.0 μmol/l for C, EC, EGCG, and ECG, and 1.56 to 50.0 μmol/l for EGC in plasma-based calibration. All reagents used were of the highest purity grade available from Sigma-Aldrich (St. Louis. MO, USA).

A complete blood count was also performed on the day of sample collection using whole blood samples anti-coagulated with EDTA. The Cell-Dyn 3200 (Abbott Laboratories, US) was used to determine the number of white blood cells (neutrophils, lymphocytes, monocytes and granulocytes) and other parameters including haemoglobin and packed cell volume. Reagents for performing complete blood count were obtained from Abbott Laboratories, USA.

Chapter Four
In Vitro Genoprotective and Damaging Effects of Green Tea: Are Effects Mediated by Dose-dependent Generation of Hydrogen Peroxide and Changes in Redox Balance?

Introduction:

Tea is the second most widely consumed beverage in the world (Cabrera *et al.,* 2006; Heiss & Heiss, 2010). There are different kinds of tea, but the most popular types are green tea, oolong tea and black tea. Among the three, green tea is reported to contain the highest levels of natural polyphenols, mainly catechins (flavan-3-ols) (Huo *et al.*, 2008). Epidemiological studies have shown that consumption of green tea is associated with lower risk of several age-related diseases, such as cancer, diabetes and cardiovascular disease (CVD) (Imai *et al*., 1997; Nakachi *et al.*, 1998; Nakachi *et al.*, 2000; Nagano *et al.*, 2001; Iso *et al.*, 2006; Kuriyama *et al.*, 2006; Panagiotakos *et al.*, 2009; Oba *et al.*, 2010; Serafini *et al.*, 2011b; Wang *et al.*, 2011). This has been thought to be due to the strong antioxidant ability of tea catechins, which constitute around 70-85% of the total polyphenols in green tea (Cabrera *et al.,* 2006; Moore *et al.*, 2009). Benzie *et al.* (1999) showed that drinking green tea increased the antioxidant capacity of human plasma within 1 hour of ingestion, showing that antioxidants in green tea are absorbed and enter the circulating plasma quickly.

Xu and colleagues (2010) have demonstrated in *in vitro* studies that pre-treatment of human retinal pigment epithelial (RPE) cells with green tea polyphenols conferred protection, in terms of cell viability, DNA fragmentation and mitochondrial dysfunction, against ultra-violet B damage (at a dose of 100μw/cm^2 for 2 hours) in a dose-dependent manner (Xu *et al*., 2010). Besides, green tea was shown to protect human osteoblasts against cigarette smoke-induced damage, but the protection was blocked by the addition of a specific haem oxygenase-1 (HO-1) inhibitor, implying that the protective effects of green tea were, at least in part, dependent upon HO-1 activity (Holzer *et al*., 2012). It is noted that HO-1 is increased as a cytoprotective response to oxidative stress (Benzie and Wachtel-Galor, 2010). Therefore, this finding suggests that green tea may not act simply or solely through direct antioxidant effects but that other mechanisms are important (Holzer *et al*., 2012). In addition to *in vitro* studies, animal studies have also shown that green tea could help lower oxidative stress by decreasing DNA strand breaks or oxidative lesions, as assessed by the Comet assay (Frei & Higdon, 2003; Wong *et al*., 2005; Collins *et al*., 2008). Pre-treatment of green tea polyphenols of Imprinting Control Region (ICR) mice by gavage 2 hours before introduction of tributyltin (TBT) at a dosage of 1mg/kg significantly decreased DNA damage in a dose-dependent manner, compared to control treatment (Liu *et al*., 2008).

Interestingly, it has been reported that green tea generates hydrogen peroxide in cell culture studies, and it has been demonstrated that the generation of hydrogen peroxide was due to the auto-oxidation of catechins (Figure 4.1) (Rice-Evans *et al*., 1996; Benzie *et al*., 1999b; Chai *et al*., 2003; Halliwell & Gutteridge, 2007). A major constituent of

green tea catechins, (-)-epigallocatechin gallate (EGCG), has been shown to decrease DNA strand breaks at low concentrations but induce strand breaks at high concentrations in human lymphocytes *in vitro* (Kanadzu *et al*., 2005). Our group has shown this also with green tea (Han *et al*., 2011). These results suggest that green tea catechins may have a dual action and can be both anti- and pro-oxidant, depending on concentration. This is in agreement with the results from a previous study from our group and others which suggested there may be a pro-oxidant/ antioxidant "break-even" point for the protection/damaging action of green tea due to the hydrogen peroxide generated (Kanadzu *et al*., 2005; Han *et al*., 2011). It has been proposed by us and others that the pro-oxidant activity of green tea may also possibly be involved in triggering the indirect/adaptive antioxidant defence, via activation of the Nrf2/ARE signalling pathway, in addition to direct antioxidant protection (Benzie & Wachtel-Galor, 2010; Han *et al*., 2011).

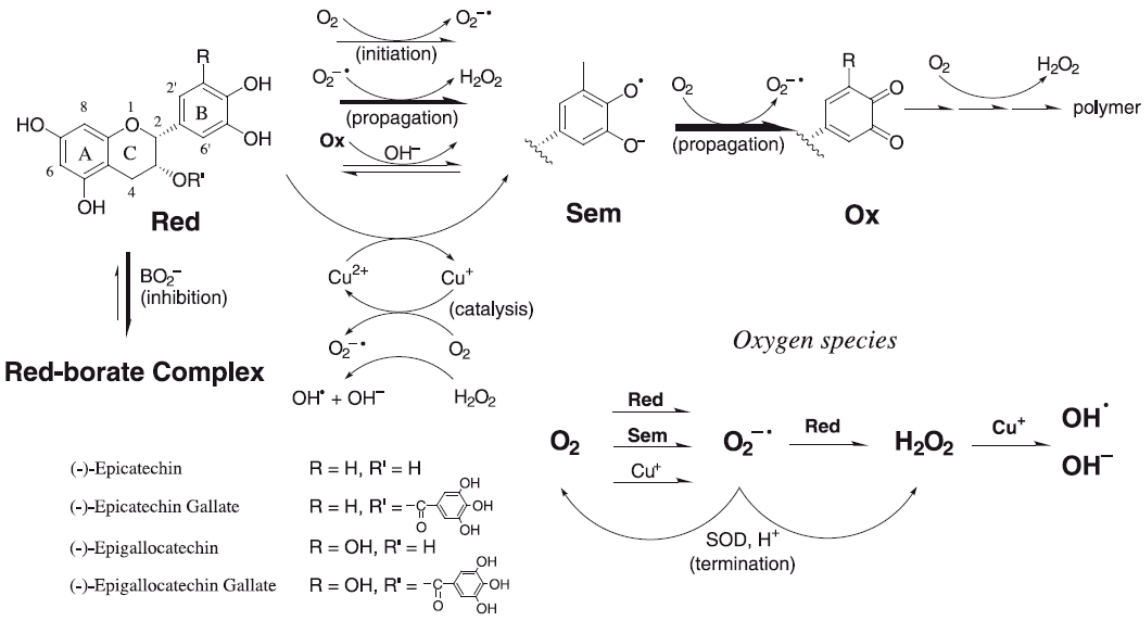


Figure 4.1 Structure and the chemical reactions of how hydrogen peroxide is produced from polyphenolic catechins (from Mochizuki *et al.*, 2002)

Hydrogen peroxide, though it is known to be a pro-oxidant contributing to oxidative stress, is an important secondary messenger in cells at low levels, i.e. at physiological levels which cells may produce themselves or obtain from exogenous sources or metabolism of xenobiotics. Hydrogen peroxide has been shown to trigger various cellular responses, such as activation of lymphocytes (Reth, 2002), increased expression of antioxidants and repair genes/proteins (Veal *et al*., 2007), and modulation of Phase II detoxification/antioxidant enzymes (Veal *et al*., 2007), to maintain normal physiological processes, and also to protect against further oxidative insults. At concentrations higher than cells can tolerate, i.e. exceeding the physiological capacity that cells can adapt to and survive, in response to hydrogen peroxide, it has been demonstrated that hydrogen

peroxide will lead to cell senescence, apoptosis and/or necrosis (Duan *et al*., 2005; Saito *et al*., 2006).

The cellular response to hydrogen peroxide results from the oxidative modification of deprotonated cysteine residues, generally known as reactive cysteines, of proteins, resulting in sulphenic acid (-SOH), disulphide bond (-S–S), sulphinic acid ($-SO_2H$) and sulphonic acid ($-SO_3H$) (Reth, 2002; Veal *et al*., 2007; Schroder & Eaton, 2008; Angeloni *et al*., 2011). The oxidative modification of transcription factors including c-Jun, Nrf2, kinases and phosphatases, will lead to a change in their activity, eliciting different cellular responses (Reth, 2002; Veal *et al*., 2007; Schroder & Eaton, 2008; Angeloni *et al*., 2011). Various phytochemicals, e.g. polyphenol-rich compounds and isothiocyanates are able to generate hydrogen peroxide in a dose-dependent manner *in vitro*, which initiates several cellular responses such as Fe(II)-dependent apoptosis in cultured cells (Nakagawa *et al*., 2004; Suh *et al*., 2010).

There is a redox-sensitive gene promoter region, the Antioxidant Response Element (ARE) also referred to as an Electrophile Response Element, with a consensus sequence "RTGACnnnGC" motif that responds to a pro-oxidant change in redox tone and that upregulates genes responsible for various cytoprotective mechanisms (Surh *et al*., 2008). Once there is a pro-oxidant shift, the transcription factor nuclear factor (erythroid-derived 2)-like 2 (NFE2L2/ Nrf2), originally located in the cytosol and kept there by a regulatory protein called Kelch-like ECH-associated protein 1 (Keap1), is released, and translocates to the nucleus. The Nrf2 will then bind to the ARE with its ARE recognition domain,

eliciting the expression of downstream cytoprotective products. These products include haem oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductases (NQO-1), glutathione-S-transferase (GSTα) and DNA repair enzymes, including hOGG1 (Halliwell & Gutteridge, 2007; Surh *et al.,* 2008; Zhang *et al.,* 2010). Increased HO-1 expression is a sign of a pro-oxidant shift, i.e. an indication of increased oxidative stress, and has cytoprotective effects, including increased ferritin expression triggered by iron produced from haem degradation in order to prevent further oxidative damage, and increased production of bilirubin, an endogenous antioxidant produced by reduction of the biliverdin that results from haem degradation (Otterbein *et al.*, 2003; Soares & Bach, 2008; Surh *et al.*, 2008; Benzie & Wachtel-Galor, 2010). Such cellular adaptations may possibly contribute to the lower levels of basal DNA damage seen after treating cells with green tea solutions or, *in vivo*, by drinking green tea, and this could be mediated by a mild tea-induced oxidative stress that, in turn, produces cytoprotective adaptations resulting in greater resistance to oxidant challenge and/or to enhanced DNA repair.

Therefore, in this part of the study, the objectives were to determine if hydrogen peroxide generated in green tea is responsible for the protective as well as the damaging effects of green tea on lymphocytic DNA and to determine whether hydrogen peroxide is involved in triggering the adaptive antioxidant response in cells to withstand a subsequent oxidant challenge.

Materials and Methods:

Experimental design:

Five apparently healthy subjects were recruited in this study with their written informed consent. Their participation was limited to supplying venous blood samples for harvesting and pooling of peripheral lymphocytes for *in vitro* study. This experiment was approved by the Human Subjects Ethics Sub-committee of The Hong Kong Polytechnic University. On the sample collection day, 20ml of heparinised venous blood was collected from all volunteers on the same day, and lymphocytes were harvested as described in Chapter 3. The washed lymphocytes from the five volunteers were pooled, aliquoted and cryopreserved. On the day of testing, lymphocyte aliquots were thawed (once only), washed and incubated in freshly prepared tea alone (with designated concentrations of 0.005%, 0.01%, and 0.05%w/v in PBS, and PBS as control) for 30 minutes at 37^{o}C in water bath, and also in another two matched sample sets, one with tea (at the same concentrations) to which catalase (CAT) had been added to remove hydrogen peroxide generated, and one with tea to which catalase+superoxide dismutase (CAT+SOD) had been added to convert superoxide to hydrogen peroxide and to remove hydrogen peroxide. The enzymes were added to the tea 5 minutes before the tea was added to the cells to ensure the removal of hydrogen peroxide in the solutions before cells came in contact with tea. Treated and control cells were washed and some cells from each treatment were used for RNA extraction to examine the change in gene expression, while others were washed and tested immediately using the Fpg-assisted version of the comet assay to assess the effect of exposure to tea on DNA damage, particularly in oxidation-induced lesions. A simplified diagram of the experimental flow is presented in Figure 4.2.

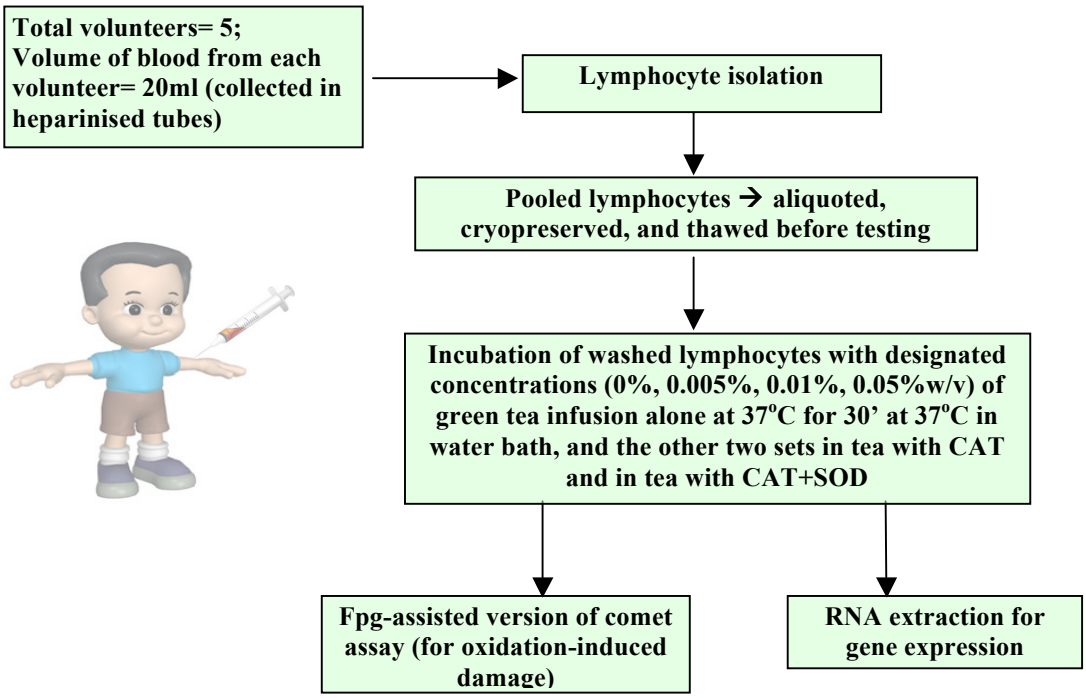


Figure 4.2 A simplified diagram showing the experimental flow of the *in vitro* green tea study

Preparation of the green tea (pre-rain Loong-cheng tea):

Pre-rain Loong-cheng tea was chosen in this study because our group has previously demonstrated the genoprotective effect of this tea on human lymphocytes *in vitro* and *in vivo* (Han *et al*., 2011). One gram of pre-rain Loong-cheng tea leaves (kindly provided by Ying Kee Tea House, HKSAR) was weighed and then brewed in 100ml boiling distilled water for 3 minutes. The tea infusion was filtered, and the prepared, filtered 1% w/v fresh pre-rain Loong-cheng tea was then diluted to concentrations of 0.005%, 0.01% and 0.05% in PBS, and 1ml of each concentration of tea solution was aliquoted into 1.5ml

microcentrifuge tubes. Freshly prepared pre-rain Loong-cheng tea solutions were used immediately in the subsequent experiments, and as described below.

Preparation of Catalase (CAT) and Superoxide Dismutase (SOD):

Stock catalase (CAT, 203U/µl) solution was first diluted with phosphate buffer (referred to simply as buffer in this chapter to avoid confusion with the buffer used in the Fpg-assisted comet assay which is referred to as enzyme buffer). The CAT solution was prepared from 50mM potassium phosphate in distilled water at pH7.0 containing 100U/µl CAT. This CAT solution was then aliquoted and stored at 4^{o}C until use, when it was further diluted 1/100 with cold buffer. The 2.4µl of this were added to 1ml aliquots of the freshly prepared tea solutions (each 1ml of tea contained 2.4U of CAT). Preliminary results showed that 2.4U CAT is sufficient to remove H_2O_2 up to a concentration of 160µM.

Stock SOD (9.6U/µl) was first aliquoted and stored at 4^{o}C until use. The aliquot of SOD was diluted with buffer to 0.96U/µl before use and a total of 4.8U was added to each aliquot of tea for the CAT+SOD set of tea solutions. The same volumes of buffer, 2.4U CAT alone+ buffer and both 2.4U CAT+ 4.8U SOD were added to three sets of freshly prepared tea solutions. CAT and SOD were obtained from Sigma-Aldrich (St. Louis, US).

Tea incubation of lymphocytes:

A cryopreserved aliquot of pooled lymphocytes was thawed and centrifuged at 1200 rpm for 5 minutes at 4^{o}C. The medium was then discarded. The cell pellet was re-suspended

with 1.2ml cold PBS, and the cell suspension and a 100µl aliquot of the cell suspension was put into each of 12 microcentrifuge tubes (3 sets of 4 tubes of cells). The cell suspension was then washed with cold PBS twice to remove any remaining freezing medium, and the supernatant was discarded after each wash. After washing, one set of cell pellets in the microcentrifuge tubes was re-suspended with 1ml PBS, 0.005%, 0.01% or 0.05% freshly prepared pre-rain Loong-cheng tea, and the other two sets with tea+ CAT and tea+CAT+SOD.

Cells were incubated in the above-mentioned concentrations of green tea at 37^{o}C for 30 minutes in a water bath. After tea incubation, lymphocytes were washed with cold PBS twice, and immediately after washing the cells were sampled from each tube and:

a) run through the Fpg-assisted comet assay to examine the Fpg-labile lesions with the established protocol (as described in detail in Chapter 3);
b) used to extract RNA to assess the change in ARE-related gene expression normalised to two lymphocyte-specific reference genes, *CD3ε* and *CD8β* (Røge *et al*., 2007). Optimised conditions and primer details are shown in Tables 4.1 and 4.2, respectively.

In addition, the hydrogen peroxide concentrations in different solutions of tea (freshly prepared and after 30 minutes at 37^{o}C) were measured as described in detail in Chapter 3. Details for performing the Fpg-assisted comet assay and gene expression can be found in Chapter 3.

Statistical analysis:

One-way ANOVA with Bonferroni's post-hoc test was conducted on the hydrogen peroxide concentrations in different concentrations of tea before and after 30 minutes incubation. Three independent experiments were performed with the pooled lymphocytes. In the Fpg-assisted comet assay, two gels for each treatment were prepared to score DNA damage (presented as %DNA in tail), and a total of 50 nucleoids was scored in each single gel. Repeated-measures ANOVA, with Bonferroni's post-hoc test was performed for the examination of changes in oxidation-induced lesions in lymphocytic DNA with tea pre- treatment. The data for the hydrogen peroxide concentrations in tea and the Fpg-assisted comet assay are expressed as mean (SD); $p<0.05$ was considered to be statistically significant.

An open access gene analysis software tool, Relative Expression Software Tool (REST© 2009, Qiagen), was used to analyse data obtained from real time PCR (Pfaffl *et al*., 2002). This software takes into account the PCR efficiencies of genes of interest and reference genes, and is able to use more than one reference gene for normalisation to increase the reliability of the results. The statistical software uses randomisation and bootstrapping methods to test for the statistical significance of the expression ratios determined. The data for gene expression are presented in box and whisker plots; $p<0.05$ was considered to be statistically significant, whereas fold change of>2, i.e. 2-fold, was considered as a biologically meaningful change in relation to analysing gene expression data (King *et al*., 2001; Zajchowski *et al*., 2001; Tu *et al*., 2002).

Table 4.1. Preparation of master mix for real time PCR

a) Composition of master mix for a ***single*** reaction.

Reagents	Volume (µl)	Final concentration
10X PCR Buffer	1.0	/
2mM dNTP	1.0	0.1mM
10µM Forward primer	0.3	300nM
10µM Reverse primer	0.3	300nM
25mM $MgCl_2$	Primer dependent	Primer dependent
100X SYBR® Green	0.04	0.4X
5µM ROX®	0.04	20nM
MilliQ water	Make up to 9µl	/
5U/µl AmpliTaq Gold®	0.24	1.2U

b) $MgCl_2$ concentrations used for each pair of primers:

[$MgCl_2$] (mM)	Primer used	Volume added (µl)/ Rx
2.5	*CD8β, KEAP1*	1.0
3	*CD3ε, HMOX1, NRF2, BACH1, hOGG1*	1.2

Table 4.2. Primers for each gene of interest

Gene	Primers	Sequence	Size (bp)
CD3ε	Forward	5′-GGCAGGCAAAGGGGACA-3′	236
	Reverse	5′-ACCATGAGGCTGAGGAACGAT-3′	
CD8β	Forward	5′-GACAGTCACCACGAGTTCCT-3′	133
	Reverse	5′-GCTTCACGCTTGTGAGATTGAGAA-3′	
HMOX1	Forward	5′-CAGGCAGAGAATGCTGAGTTC-3′	146
	Reverse	5′-GATGTTGAGCAGGAACGCAGT-3′	
NRF2	Forward	5′-AGTGGATCTGCCAACTACTC-3′	106
	Reverse	5′-CATCTACAAACGGGAATGTCTG-3′	
KEAP1	Forward	5'-ATGCCTCAGTGTTAAAATGACAT-3'	224
	Reverse	5'-CAGGTATCCAAGAATAAATCACA-3'	
BACH1	Forward	5′-TGCGATGTCACCATCTTTGT-3′	100
	Reverse	5′-CCTGGCCTACGATTCTTGAG-3′	
hOGG1	Forward	5′-ACACTGGAGTGGTGTACTAGCG-3′	301
	Reverse	5′-GCCGATGTTGTTGTTGGAGG-3′	

Results:

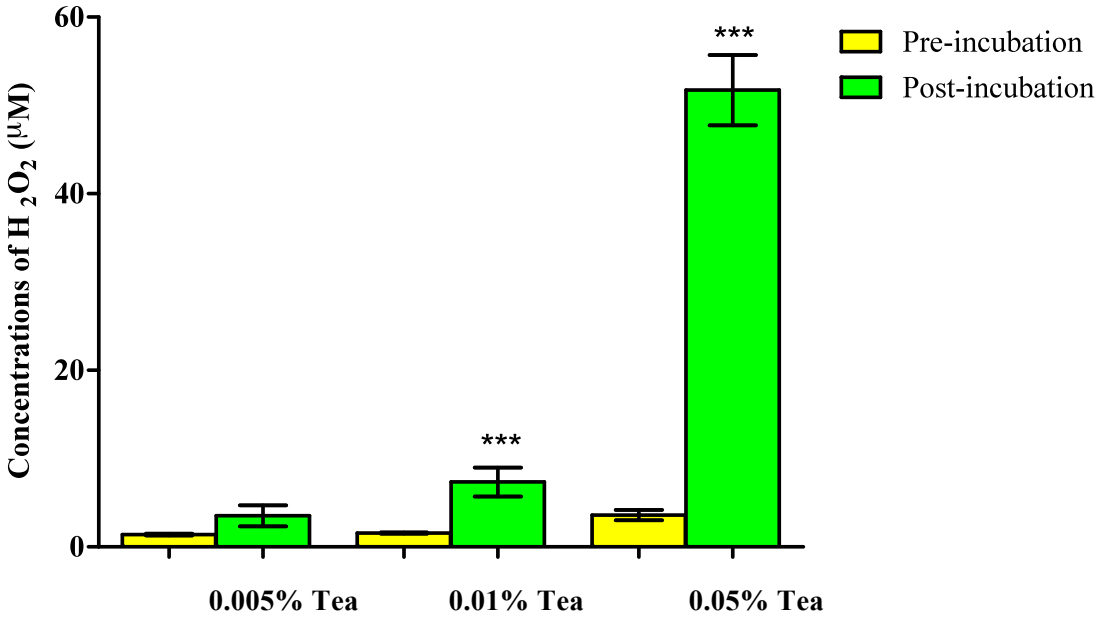
H_2O_2 in green tea solutions before and after 30 minutes incubation at 37°C:

Significant amounts of hydrogen peroxide were generated in the green tea solutions, and in a dose-dependent manner, during 30 minutes incubation. Data are shown as Mean (SD) in Table 4.3 and in Figure 4.3.

Table 4.3 Concentrations of H_2O_2 measured in tea infusions before (freshly prepared) and after incubation at 37°C in water bath for 30min. Data are presented as mean (SD), n=3, with PBS as blank; *** $p<0.001$ compared to the fresh tea solution of the same concentration

Tea solution (% w/v in PBS)	**0.005%**	**0.01%**	**0.05%**
Concentration of H_2O_2 (μM) *Pre-incubation* *(Fresh infusion)*	1.41 (0.10)	1.57 (0.07)	3.61 (0.58)
Concentration of H_2O_2 (μM) *Post-incubation* *(After 30 min at 37°C)*	3.52 (1.18)	7.36 (1.64)***	51.73 (3.98)***

Figure 4.3 Concentrations of H_2O_2 generated in tea infusions (prepared in PBS) before and after incubation at 37°C for 30min. Data are presented as mean±SD, n=3 with PBS as blank; ***: $p<0.001$ compared to tea solution of the same concentration before incubation



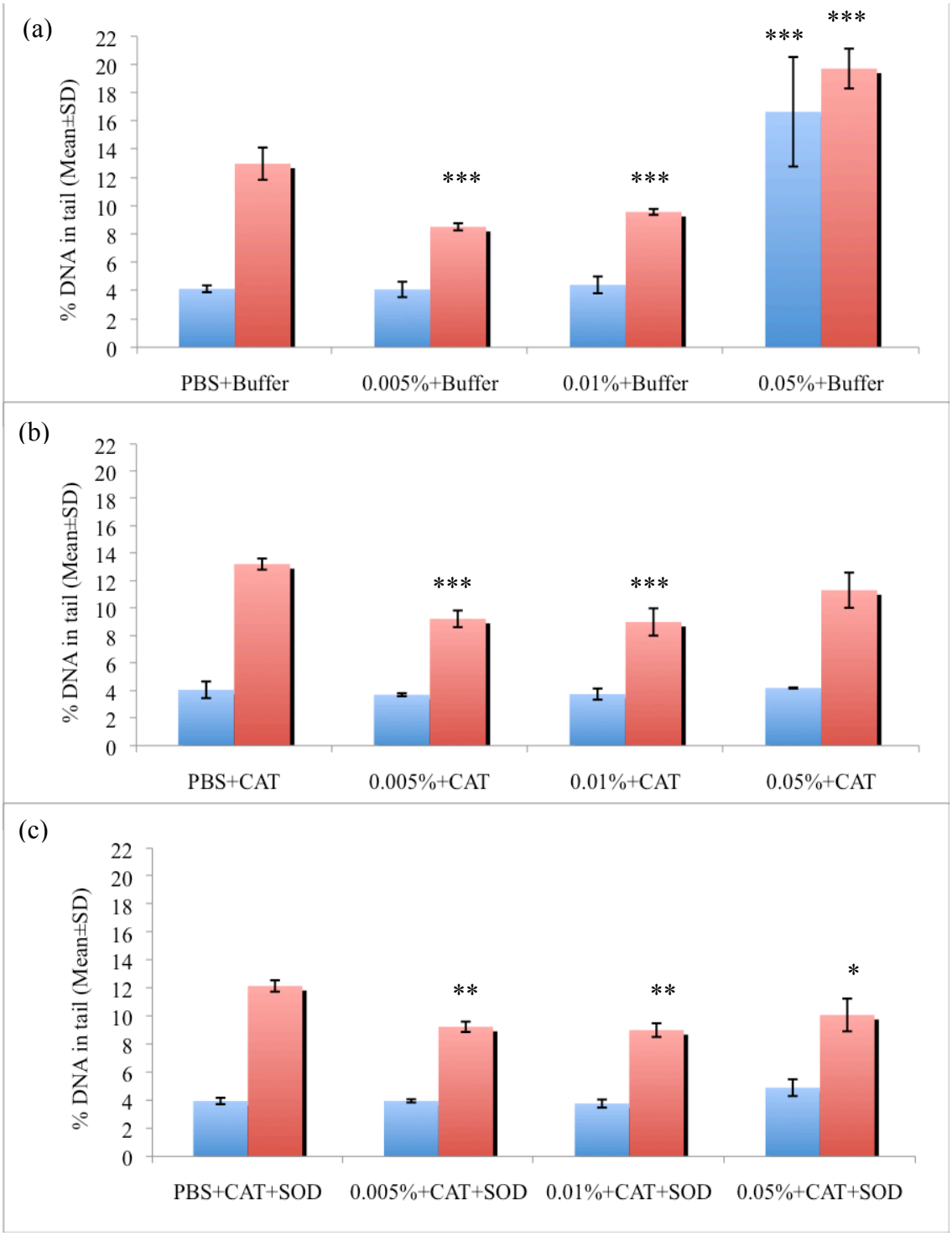
DNA damage in pooled human lymphocytes treated *in vitro* with different concentrations of green tea solutions with/without catalase (CAT) or catalase+superoxide dismutase (CAT+SOD)

As shown in Table 4.4 and Figure 4.4, treatment of cells with 0.05%w/v tea caused a 4-fold increase ($p<0.001$) in single strand breaks (the Fpg buffer treated cells, but without Fpg) compared to PBS treated cells. This damage was prevented by the presence of CAT or CAT+SOD in the tea. No increase in single strand breaks was seen in the cells pre-treated with 0.005% or 0.01% tea, even in the absence of CAT or CAT+SOD. With regard to the Fpg comet assay results, there were significantly ($p<0.001$) lower DNA damage scores in cells pre-treated with 0.005% and 0.01% green tea alone (i.e. no CAT or SOD) compared to cells pre-treated with PBS. However, the DNA damage in cells pre-treated with 0.05% green tea was significantly ($p<0.001$) higher than in control cells and in cells pre-treated with the lower concentrations of green tea. In the cells pre-treated with tea in the presence of CAT or CAT+SOD, the damaging effect of the 0.05% concentration of tea was not seen. However, the 0.005% and 0.01% tea treated cells still showed significantly less ($p<0.001$) DNA damage even when CAT or CAT+SOD was present.

Table 4.4 showing the %DNA in the tail of both Fpg-buffer and Fpg-treated cells after treating cells with tea with PBS, with CAT, and with CAT+SOD at 37^{o}C for 30min. Data are presented as mean (SD), n=3; *p<0.05, **p<0.01, ***p<0.001 compared to tea solutions of other concentrations and PBS control in the same treatment set

	%DNA in comet tail [Mean (SD)]											
Treatment	+Buffer				+CAT				+CAT+SOD			
	PBS	0.005% Tea	0.01% Tea	0.05% Tea	PBS	0.005% Tea	0.01% Tea	0.05% Tea	PBS	0.005% Tea	0.01% Tea	0.05% Tea
Buffer-treated (no Fpg) gels	4.14 (0.24)	4.09 (0.55)	4.42 (0.59)	16.66*** (3.88)	4.08 (0.61)	3.71 (0.11)	3.76 (0.41)	4.21 (0.04)	3.96 (0.23)	3.96 (0.12)	3.77 (0.29)	4.91 (0.60)
Fpg-treated gels	12.99 (1.14)	8.52*** (0.25)	9.57*** (0.21)	19.72*** (1.41)	13.24 (0.41)	9.25*** (0.99)	9.02*** (0.99)	11.24 (1.28)	12.17 (0.41)	9.26** (0.37)	9.02** (0.49)	10.10* (1.17)

Figure 4.4 DNA damage (as %DNA in comet tail) after treating lymphocytes for 30min *in vitro* in (a) tea + PBS; b) tea + CAT; c) tea + CAT+SOD. Data are presented as mean±SD, n=3; Blue column: Fpg-buffer-treated cells, Red column: Fpg-treated cells. *p<0.05, ** p<0.01, ***p<0.001 compared to PBS control cells of the same set



Fold change in gene expression level of ARE-related genes in pooled human lymphocytes treated with different concentrations of green tea infusion with and without CAT and CAT+SOD

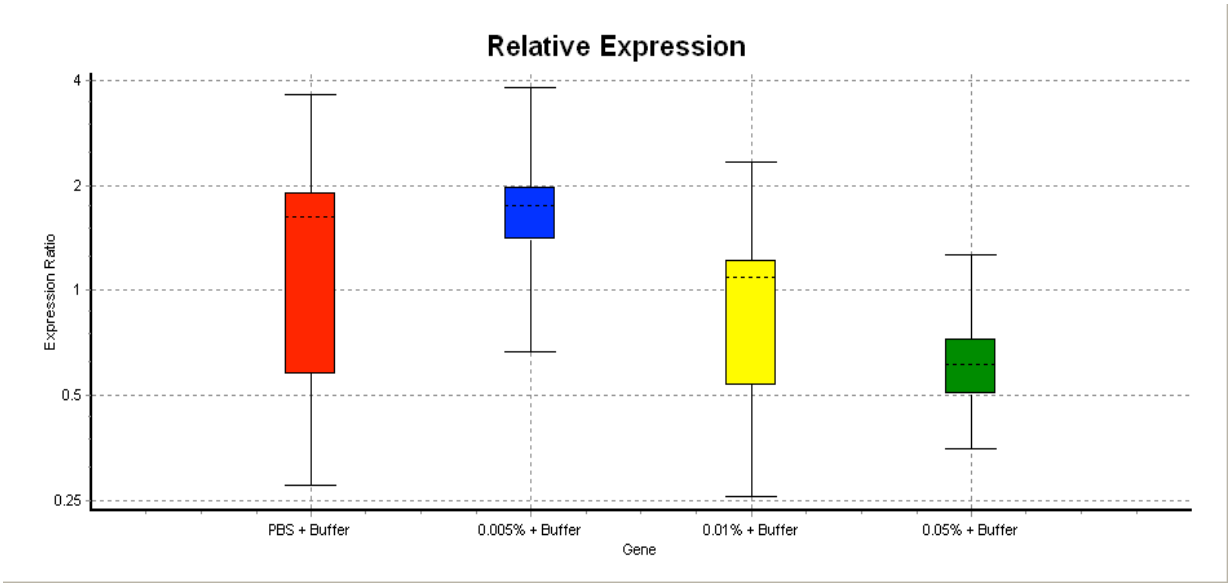
Two reference genes, *CD3ε* and *CD8β* were applied to normalise the results obtained. REST© 2009 was used for statistical analysis where randomisation and bootstrapping methods were applied to test for the statistical significance ($p<0.05$) of the expression ratios determined.

No significant changes in the expression ratio of any of the ARE-related genes of interest, i.e. *HMOX1* (Figure 4.5a), *NRF2* (Figure 4.5b), *KEAP1* (Figure 4.5c), *BACH1* (Figure 4.5d), and *hOGG1* (Figure 4.5e), were observed in lymphocytes pre-treated with different concentrations of tea compared to PBS treatment.

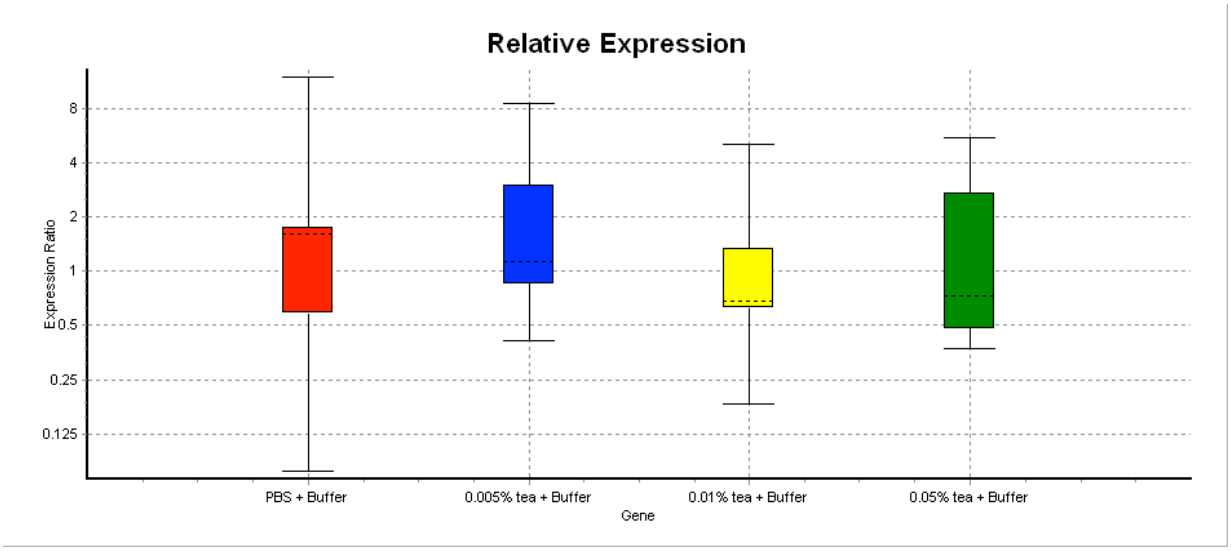
Addition of CAT to remove hydrogen peroxide in tea did not significantly change the expression ratios of the ARE-related genes, *HMOX1* (Figure 4.6a), *NRF2* (Figure 4.6b), *KEAP1* (Figure 4.6c), *BACH1* (Figure 4.6d), and *hOGG1* (Figure 4.6e), at different concentrations of tea solutions tested. Similarly, no changes were observed with tea+ CAT or CAT+SOD for *HMOX1* (Figure 4.7a), *NRF2* (Figure 4.7b), *KEAP1* (Figure 4.7c), *BACH1* (Figure 4.7d), and *hOGG1* (Figure 4.7e).

Figure 4.5 Effects of tea incubation on expression of ARE-related genes in lymphocytes pre-treated with tea (0.005%, 0.01% and 0.05%), at 37^{o}C for 30min; (a) *HMOX1*, (b) *NRF2*, (c) *KEAP1*, (d) *BACH1*, (e) *hOGG1*, using both *CD3ε* and *CD8β* as reference genes. Data are presented as box and whisker plots, with whiskers showing the range, the box represents the lower and upper quartiles, and the dotted line in the box representing the median (n=3)

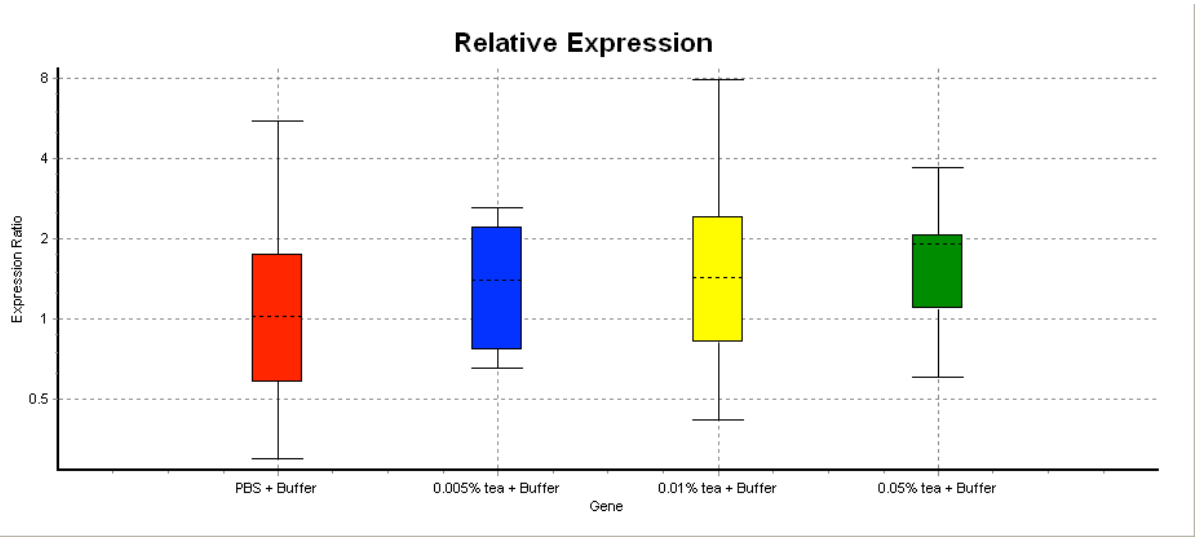
(a) *HMOX1*



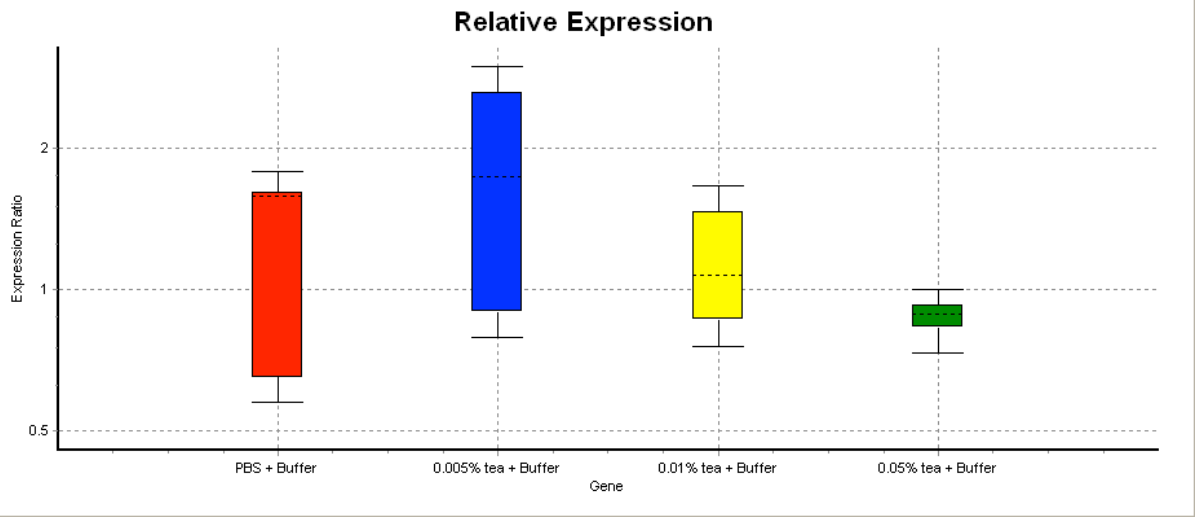
(b) *NRF2*



(c) *KEAP1*



(d) *BACH1*



(e) *hOGG1*

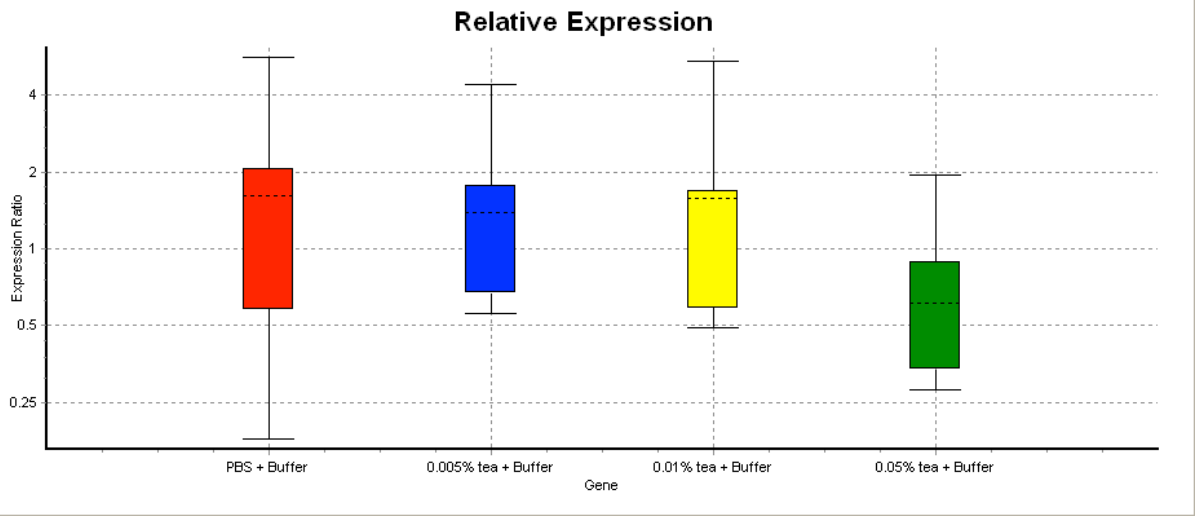
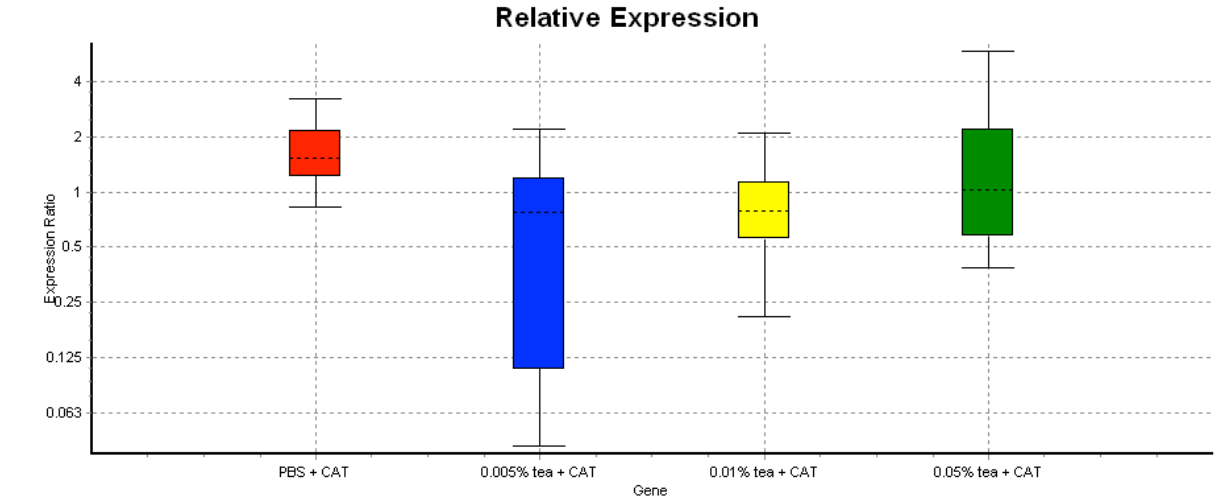
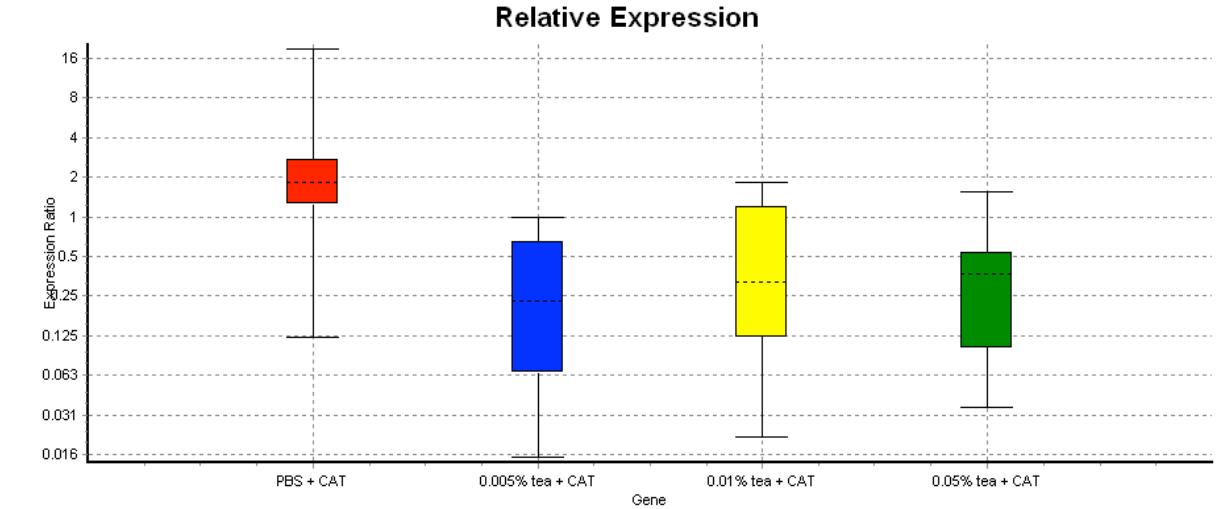


Figure 4.6 Effects of tea incubation on expression of ARE-related genes in lymphocytes pre-treated for 30min at 37°C with tea (0.005%, 0.01% and 0.05%,) containing catalase at 37°C for 30min; (a) *HMOX1*, (b) *NRF2*, (c) *KEAP1*, (d) *BACH1*, (e) *hOGG1*, using both *CD3ε* and *CD8β* as reference genes. Data are presented as box and whisker plots, with whiskers showing the range, the box represents the lower and upper quartiles, and the dotted line in the box representing the median (n=3)

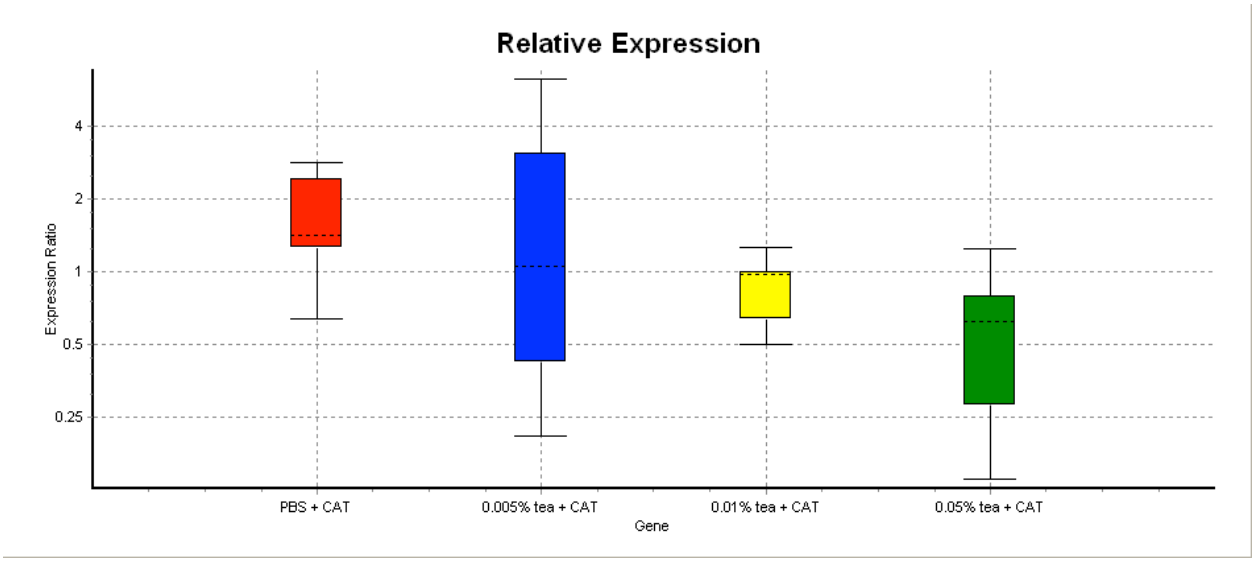
(a) *HMOX1*



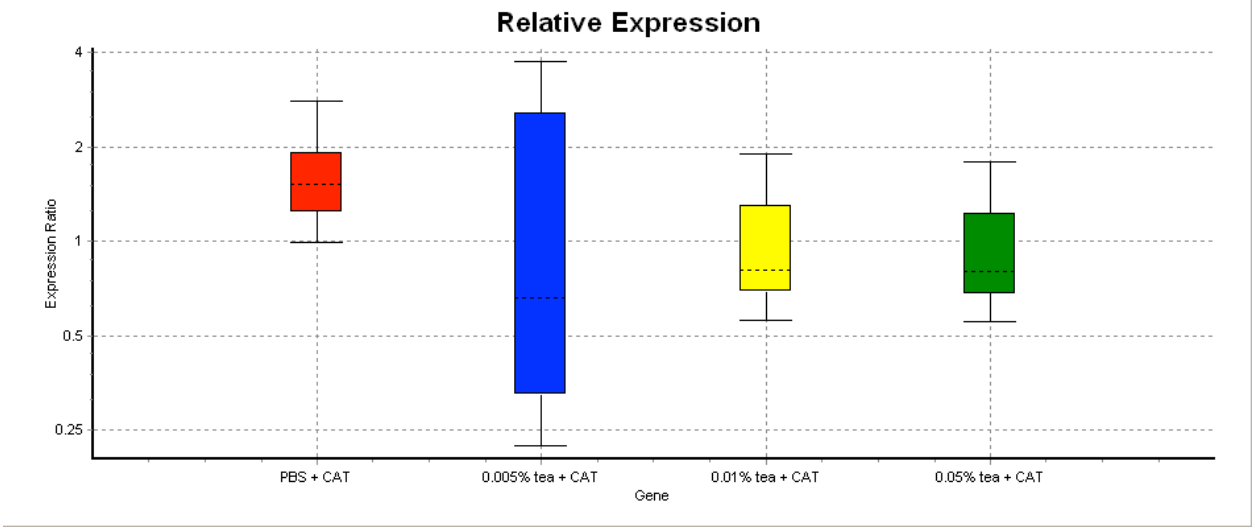
(b) *NRF2*



(c) *KEAP1*



(d) *BACH1*



(e) *hOGG1*

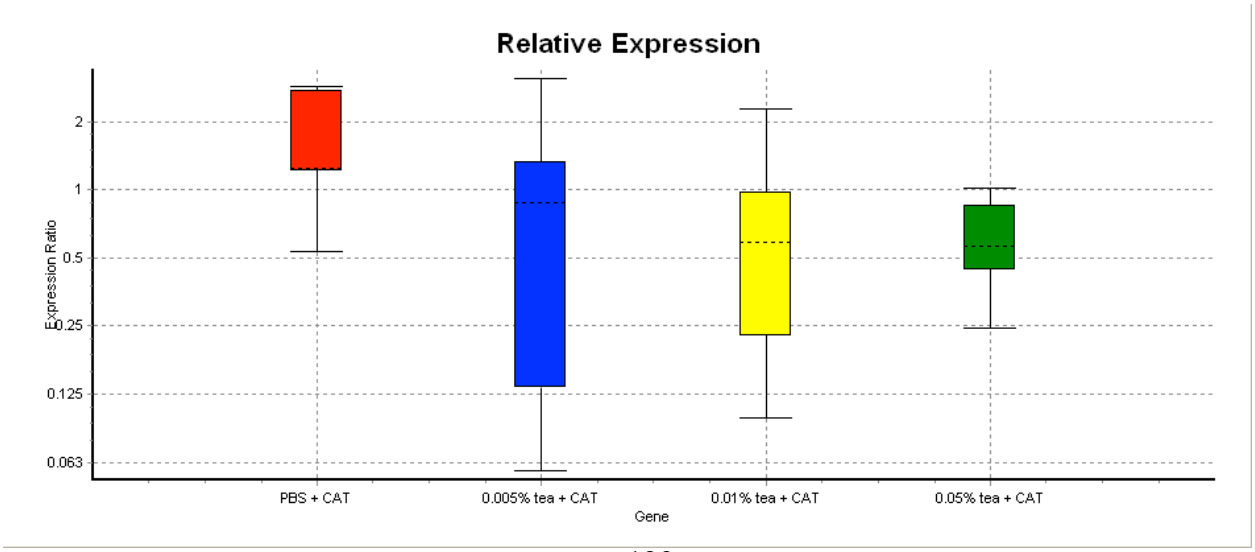
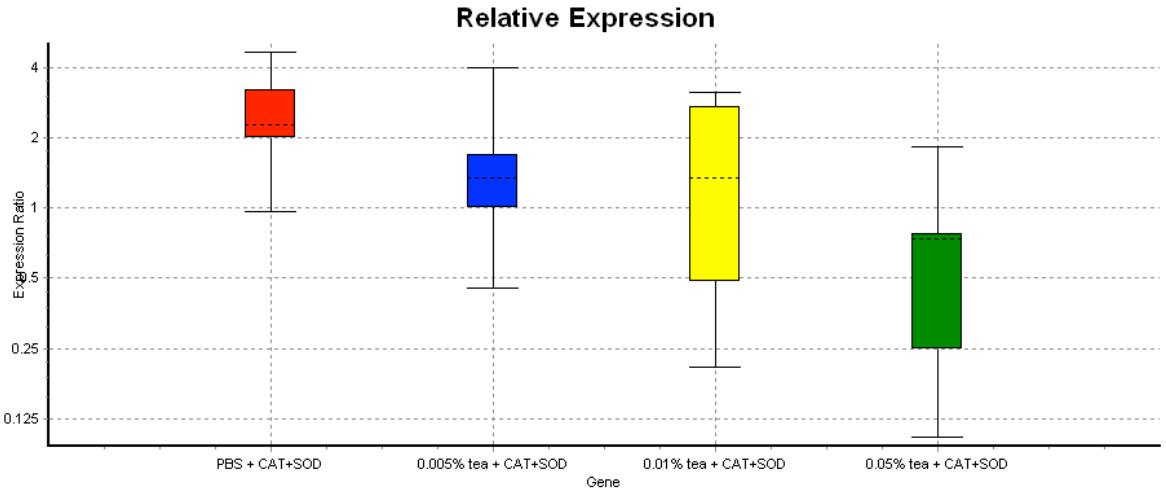
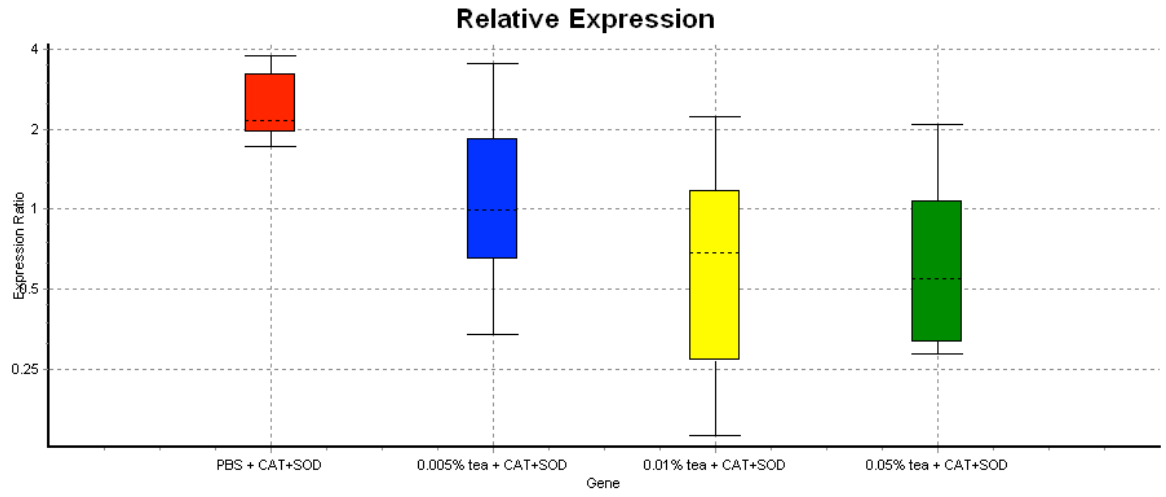


Figure 4.7 Effects of tea incubation on expression of ARE-related genes in lymphocytes pre-treated for 30min at 37°C with tea (0.005%, 0.01% and 0.05%,) containing catalase and superoxide dismutase at 37°C for 30min; (a) *HMOX1*, (b) *NRF2*, (c) *KEAP1*, (d) *BACH1*, (e) *hOGG1*, using both *CD3ε* and *CD8β* as reference genes. Data are presented as box and whisker plots, with whiskers showing the range, the box represents the lower and upper quartiles, and the dotted line in the box representing the median (n=3)

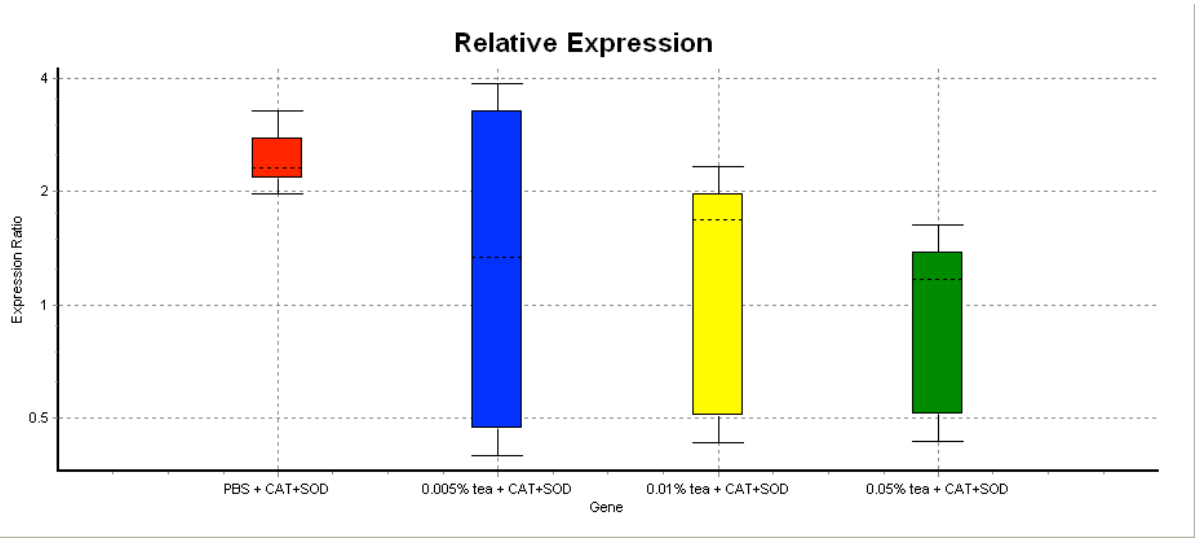
(a) *HMOX1*



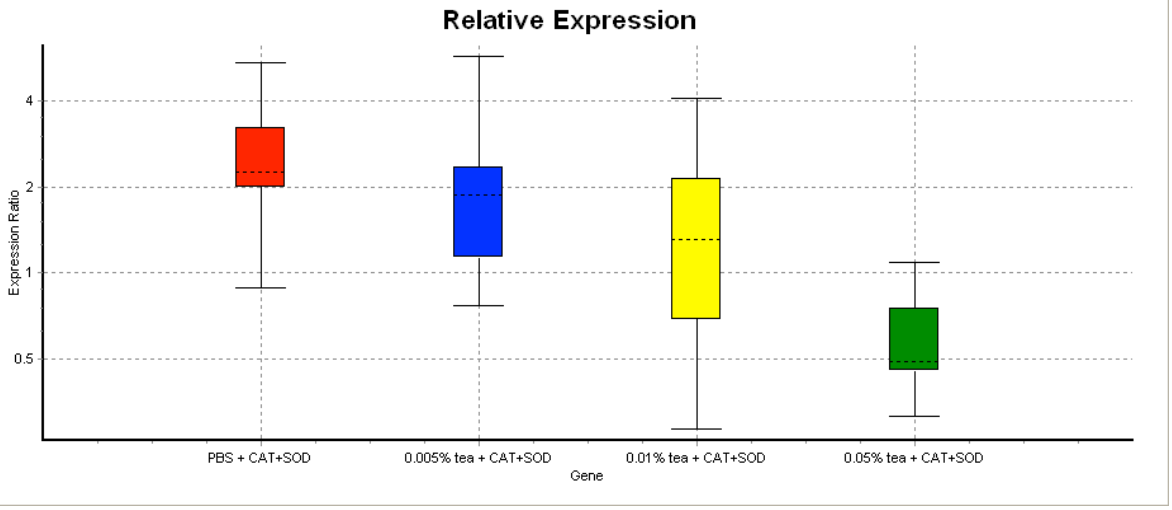
(b) *NRF2*



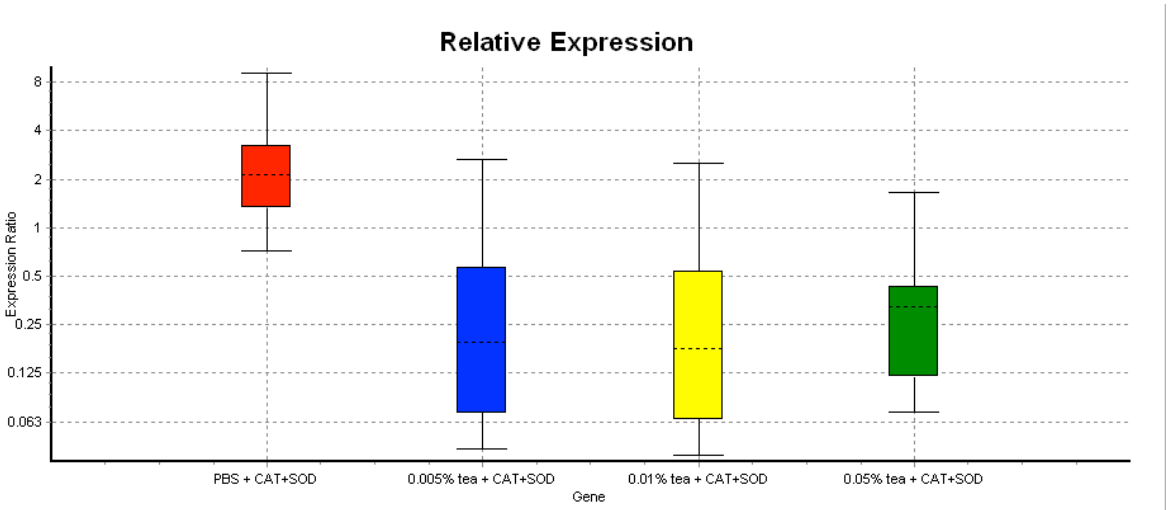
(c) *KEAP1*



(d) *BACH1*



(e) *hOGG1*



Discussion:

Drinking green tea has been implicated in lowered risk of degenerative diseases, such as diabetes and cardiovascular diseases, and this is suggested to be due to the antioxidant effects of its major components, the polyphenolic catechins (Cabrera *et al*., 2006; Moore *et al*., 2009). Interestingly, studies have shown that hydrogen peroxide, a known oxidant, is generated from green tea *in vitro* (Chai *et al*., 2003; Halliwell & Gutteridge, 2007). It has not yet been confirmed that hydrogen peroxide is also generated *in vivo* after drinking green tea, but it has been demonstrated that hydrogen peroxide concentrations are elevated in urine collected two hours after drinking green tea (Hiramoto *et al*., 2002).

Hydrogen peroxide generation in green tea may be involved in triggering intracellular signalling, and thereby initiating the adaptive cellular response for cytoprotection through up-regulation of ARE-driven genes, e.g. haem oxygenase 1 (encoded by *HMOX1*). The direct antioxidant and adaptive protection mechanisms may act in concert to achieve the beneficial effects of green tea (Benzie & Wachtel-Galor, 2010; Han *et al*., 2011). Our previous studies have shown that drinking green tea increases plasma antioxidant capacity rapidly and that regular intake provides genoprotective effects (Benzie *et al*., 1999b; Han *et al*., 2011). However, it was unclear if the genoprotective effect of green tea on lymphocytic DNA was caused by the direct antioxidant effect or by an adaptive response via hydrogen peroxide generated from green tea. To examine the possible mechanism of green tea for genoprotective effects, by either directly acting as an antioxidant or by eliciting an adaptive response in cells via hydrogen peroxide or both,

this *in vitro* study treated pooled lymphocytes from apparently healthy volunteers with different doses of tea in the presence and absence of CAT and CAT+SOD.

Results showed genoprotective effects of green tea at low concentrations (0.005% and 0.01%w/v) in the absence of CAT or CAT+SOD. This is consistent with our previous findings (Han *et al*., 2011). However, a DNA damaging effect was observed with 0.05% tea solution. Animal studies have found that a high dose of green tea polyphenols, e.g. >200μM EGCG or >500mg/kg given via intra gastric tube, induce hepatotoxicity, nephrotoxicity and even death in mice and dogs (Galati *et al*., 2006; Lambert *et al*., 2010; Martin & Appel, 2010; Inoue *et al*., 2011). It is possible that these toxic effects are due to generation of hydrogen peroxide. The green tea used in this study was shown to generate hydrogen peroxide in a dose-dependent manner, and the concentrations of hydrogen peroxide were significantly increased in 0.01% and 0.05% tea solutions, from 1.6μM to 7.6μM and from 3.6μM to 51.7μM, respectively, after incubation at 37^{o}C for 30 minutes. In general, challenging lymphocytes with hydrogen peroxide at 30μM on ice for five minutes is sufficient to induce increased DNA damage to around 25% in terms of %DNA in tail (Han *et al*., 2011; our unpublished data).

Compared to DNA damage induced by 30μM hydrogen peroxide, the DNA damage caused by 0.05% tea solution was around 19%. The difference is possibly due to the gradual increase in hydrogen peroxide content in the solution over the 30 minutes incubation time. Interestingly, addition of CAT or CAT+SOD eliminated the increased DNA damage observed after incubation with 0.05% tea. This showed that hydrogen

peroxide generated in the 0.05% tea solution was responsible for the damaging effect seen in cells incubated for 30 minutes in 0.05% tea. However, addition of CAT or CAT+ SOD did not affect the genoprotective effect seen in the 0.005% and 0.01% tea-treated cells, implying that, under the conditions used, protection of lymphocytic DNA was not a result of a mild oxidative stress induced by generation of hydrogen peroxide. In other words, the relatively large amount of hydrogen peroxide generated by 0.05% tea was sufficient to induce DNA damage, and was prevented by removal of hydrogen peroxide, but the genoprotection seen with lower concentrations of tea was not prevented by removal of hydrogen peroxide.

It has been hypothesised that hydrogen peroxide acts as a secondary messenger to induce cytoprotection via ARE-driven genes. This was investigated by looking at the expression of several of these genes in the cells pre-treated with tea in the presence and absence of CAT. However, no increased expression of any genes was observed. It is possible that, ARE-related cytoprotective genes may have been activated, but have not had time to become expressed at measurably greater levels. A longer incubation time might be needed. However, on the basis of the gene expression data, green tea does not confer acute protection to lymphocytic DNA through adaptive cytoprotection involving the triggering of the Nrf2/ARE signalling pathway. Nonetheless, genoprotection was seen in cells pre-treated with green tea a low doses with and without CAT or CAT+SOD. Direct antioxidant effects of green tea polyphenols contributing to the protective effects observed at low concentrations of green tea would be a possible explanation for this observation. It is also possible that green tea may help increase the activity of the DNA

repair enzyme, hOGG1, by blocking the actions of its inhibitors, e.g. protease. It is noted that no change in *hOGG1* expression was observed, but increased activity of the protein would lower DNA damage.

Fpg is specific for oxidised purines, including 8-oxo-7,8-dihydroguanine (8-oxoGua), 2,6-diamino-4-hydroxy-5-formamido- pyrimidine (FaPyGua) and 4,6-diamino-5-formamidopyrimidine (FaPyAde) and other ring-opened purines. The enzyme is a microbial analogue of hOGG1, which catalyses the first step in base excision repair (Collins & Azqueta, 2012). However, it is possible that other DNA repair enzymes responsible for the elimination of oxidation-induced DNA damage, e.g. MYH encoding A/G-specific adenine DNA glycosylase, NTH1 encoding for endonuclease III, and MTH1 encoding for 8-oxo-dGTPase, may be triggered by green tea (Koketsu *et al*., 2004; Zhang *et al*., 2005).

Several studies have examined the effects of time-dependent change in the expression of ARE-related genes under various stimulations, e.g. cigarette smoke in mouse fibroblasts, sulphoraphane in human astrocytes and arsenite in human keratocytes (Bosio *et al*., 2002; Reichard *et al*., 2007; Bergstrom *et al*., 2011). The change in *HMOX1* gene expression was observed after >1 hour stimulation (Bosio *et al*., 2002; Reichard *et al*., 2007; Bergstrom *et al*., 2011) It is worth noting that Bosio and co-workers demonstrated that *HMOX1* expression in the mouse fibroblast cells was not significantly changed 0.5 hours after exposure to cigarette smoke-infused PBS, but was time-dependently upregulated after exposing cells to cigarette smoke-infused PBS for 1-8 hours (Bosio *et al*., 2002).

This supports the suggestion that a longer time of tea-incubation may be needed to reveal changes in gene expression of ARE-regulated genes. Besides, flavonoids, the polyphenolic group of compounds to which green tea catechins belongs, have also been suggested to be signalling molecules, directly triggering cytoprotection as opposed to cytoprotection being solely initialised by hydrogen peroxide (Williams *et al*., 2004; Peer & Murphy, 2006). It would be worthwhile to further investigate the effect of longer incubation time of lymphocytes in tea solutions in the presence and absence of CAT.

It has also been suggested that the pH of the *in vitro* system used may lead to the difference in pro-oxidative/ anti-oxidative nature of polyphenols. Polyphenols were shown to be pro-oxidants at pH 7.4 but antioxidants at pH 5.4 or below (Akoh & Min, 2003; Edeas, 2011). The strong buffering activity in blood and tissues may add an extra layer of complexity to the effects of consumption of green tea *in vivo*, as it is known that pH of different tissues and the status of the tissue, i.e. normal or cancerous, varies (Gerweck & Seetharaman, 1996). Therefore, it is possible to speculate that green tea may exert tissue-specific pro-oxidative or antioxidative actions, and this may be direct or mediated via hydrogen peroxide. Further *in vivo* studies are required to provide more insight into the direct and indirect antioxidant effects of green tea for protection against oxidative insults.

In conclusion, a direct antioxidant effect of green tea against ubiquitous ROS in the *in vitro* testing system used could have been responsible for the genoprotective effects observed with the low doses of tea, while the large amount of hydrogen peroxide

generated over the 30 minutes in the high dose of tea solution was responsible for the DNA damage caused. While the damage was mitigated by CAT and CAT+SOD, no evidence of adaptive cytoprotective responses triggered was seen in terms of expression of redox sensitive genes. A longer incubation time, to allow adequate expression of the relevant genes, may be required. Ideally, expression of these genes, along with DNA damage, should be explored *in vivo* and in human trials. This was the focus of the next part of the study.

Chapter 5
Effects of Acute and Short-term Green Tea Ingestion on Redox Balance, DNA Damage & Repair in a Randomised, Controlled Supplementation Study

Introduction:

Oxidative stress has been reported to increase during ageing, and this increase is associated with several age-related diseases, such as cardiovascular diseases, cancer and diabetes (Rahman & MacNee, 2000; Szeto & Benzie, 2002; Williamson *et al.*, 2009). Consumption of tea has been associated with decreased risk of age-related diseases (Cabrera *et al.,* 2006; Moore *et al.*, 2009). Tea, particularly green tea, is rich in polyphenols, the flavan-3-ols, because of the inactivation of polyphenol oxidase during processing of the freshly picked tea leaves. Polyphenols have been reported to protect against oxidative stress (Hanhineva *et al.*, 2010). *In vitro* and animal studies have demonstrated beneficial effects of green tea, such as promotion of apoptosis of cancer cells and alleviation of inflammation (Khan & Mukhtar, 2007; Boehm *et al.*, 2009; Moore *et al.*, 2009). Many epidemiological studies on green tea consumption and risk of various diseases, in particular in relation to cancer risk, have been conducted in different continents, countries and amongst different races, but the results have been inconsistent, possibly due to the variations in tea production and preparation, genetics, lifestyle and the environmental exposures experienced by the study populations (Boehm *et al.*, 2009).

Though epidemiological studies have been inconclusive with regard to the beneficial effects of green tea consumption, evidence of positive health-related outcomes in human randomised clinical trials, e.g. increased resistance to oxidative stress in healthy individuals, better glycaemic control, decreased lipid peroxidation in patients with impaired glucose tolerance, and improvement in endothelial cell function and flow-mediated endothelium dependent vasodilation of brachial artery in smokers, have been reported (Kim *et al*., 2006; Fukino *et al*., 2008; Fenercioglu *et al*., 2010). Our group conducted a controlled human intervention study which demonstrated a genoprotective effect of drinking green tea for 30 days. In that study, increased resistance of lymphocytes to oxidant challenge and a decrease in oxidation-induced lesions in lymphocytic DNA were seen (Han *et al*., 2011).

Beneficial effects of green tea consumption are thought to be a result of the robust antioxidant ability of green tea. However, green tea generates hydrogen peroxide, which appears to be contradictory to this idea (Chai *et al.*, 2003; Halliwell, 2007b; Aoshima, 2008; Lambert & Elias, 2010). It has been suggested therefore, that green tea may act through other mechanisms to exert the reported beneficial effects, in addition to scavenging free radicals via direct antioxidant protection (Khan *et al.*, 2006; Wu *et al.*, 2006; Ogborne *et al.*, 2008; Sahin *et al*., 2010). The generation of hydrogen peroxide from tea polyphenols has only been shown *in vitro* and it is not known if this occurs *in vivo*, but this opens up the possibility that dietary antioxidants, such as green tea polyphenols, could induce a mild pro-oxidant effect on cellular redox balance that could cause the cell to produce cytoprotective elements that could, for example, increase the

activity of DNA repair enzymes and/or up-regulate the expression of endogenous antioxidants. Since there is little evidence from human studies showing the molecular effects of phytochemicals, in particular green tea, in relation to redox shift and cell signalling, *in vitro* studies have contributed to a significant body of knowledge in this area. Cytoprotection has been reported to be triggered by treatment with phytochemicals such as green tea, possibly via a change in redox balance with activation of the Nrf2/ARE signalling pathway (Wu *et al.*, 2006; Ogborne *et al.*, 2008; Benzie & Wachtel-Galor, 2010; Sahin *et al*., 2010; Zhang *et al.*, 2010). By binding to the Antioxidant Response Element (ARE) in the nucleus after a pro-oxidant stimulus induced its release in the cytosol, a redox sensitive transcription factor, nuclear factor erythroid 2 p45 (NF-E2)-related factor (Nrf2), regulates the expression of phase II detoxifying enzymes, the DNA repair enzyme hOGG1, haem oxygenase-1, and several endogenous antioxidants, for example, glutathione S-transferase (GST), glutathione peroxidase (GPx), and glutamate cysteine ligase (GCL) (Halliwell & Gutteridge, 2007; Surh *et al.,* 2008; Zhang *et al.,* 2010). Sriram and colleagues (2008) reported the restoration of activities of phase II detoxifying and antioxidant enzymes and the upregulation of Nrf2 expression at both gene and protein levels after EGCG treatment (20mg/kg intraperitoneally for 28 days) in Wistar rats with bleomycin-induced pulmonary fibrosis. It has been speculated that the effect was due to the possible interaction between EGCG and Keap1 which promoted the dissociation of Nrf2 from Keap1, although Keap1 expression was not found to be altered in this study (Sriram *et al.*, 2008).

Haem oxygenase-1 (HO-1) is one of the major products of the Nrf2/ARE signalling pathway (Immenschuh & Ramadori, 2000; Otterbein *et al.*, 2003; Calabrese *et al.*, 2009). Haem oxygenase-1 is responsible for haem degradation, with production of carbon monoxide, iron, and biliverdin (Immenschuh & Ramadori, 2000; Otterbein *et al.*, 2003; Calabrese *et al.*, 2009). In the last decade, HO-1 has become a therapeutic focus due to the involvement of HO-1 itself and the products resulting from haem degradation against different diseases such as cardiovascular diseases and cancer (Jozkowicz *et al*., 2007; Wang & Chau, 2010; Kim *et al*., 2011). The potential of HO-1 as a 'therapeutic funnel' has been attributed to the cytoprotective properties of its products, biliverdin-derived bilirubin and carbon monoxide (CO), which provide antioxidant and anti-inflammatory effects, respectively (Clark *et al*., 2000; Otterbein & Choi, 2000; Kapitulnik, 2004). Particularly through this cytoprotective mechanism, green tea and its catechins have been demonstrated to elicit the adaptive Nrf2/ARE response, inducing the production of HO-1. Various *in vitro* and animal studies have shown the concentration-dependent and time-dependent induction of HO-1 by green tea catechins via the upregulation of Nrf2 expression, increased nuclear translocation of Nrf2 and subsequent binding to ARE (Wu *et al*., 2006; Na *et al*., 2008; Ogborne *et al*., 2008). Increased HO-1 expression has been associated with the alleviation of clinically unfavourable situations, such as bleomycin-induced pulmonary fibrosis and cisplatin-induced nephrotoxicity in Wistar rats, and also with the protection against myocardial ischaemia-reperfusion injury (Sriram *et al*., 2009; Akhlaghi & Bandy, 2010; Sahin *et al*., 2010).

Though *in vitro* studies of phytochemicals, redox balance and cell signalling for cytoprotection have shown promising results, little research has been performed *in vivo*,

though as noted some animal studies have been performed. This is a rewarding area to investigate, though very challenging, in the area of molecular nutrition and redox balance. In this part of the study, the aim was to extend our previous work and determine if a single dose or regular but short-term (7 days) consumption of green tea by healthy subjects increases total antioxidant capacity in plasma, protects DNA against oxidation-induced lesions and increases DNA repair enzyme activity (specifically by increasing hOGG1 activity) in human subjects. Changes in gene and protein expression of selected products of ARE activation were also investigated. In addition, correlations between polyphenolic content in plasma after a single dose or regular intake of green tea and changes in the biomarkers of interest were also determined.

Materials and Methods:

Experimental design:

Sixteen apparently healthy adults (4 men and 12 women, all ethnic Chinese) aged from 35 to 50 (due to privacy concerns, some subjects did not provide their actual age, therefore, the exact mean(SD) of age could not be ascertained) were recruited from amongst the staff and fellow students at The Hong Kong Polytechnic University by word of mouth and through posters around the university. The study was approved by the Human Subjects Ethics Subcommittee of The Hong Kong Polytechnic University, and all procedures involving human subjects complied with the Declaration of Helsinki, as revised in 2008. Written consent was obtained and sample information and consent forms are provided in Appendices 1 & 2) Subjects were non-smokers with no previous history of chronic diseases such as hypertension, cardiovascular diseases and diabetes. Subjects

were not under long-term medication, had not been hospitalised in the last 12 months and had not received medical care in the past three months. Subjects who took vitamin or herbal supplements daily, had special dietary preferences, e.g. vegetarianism, or consumed green tea daily were not recruited into the study. Subjects with Body Mass Index (BMI) higher than 27kg/m^2 (regarded as obese according to American Asian Diabetes Society) were also excluded.

Subjects were non-selectively assigned to take either tea (n=8) or water (n=8) first in this single-blinded, placebo-controlled cross-over trial. On day 1 of each subject's participation, baseline fasting blood and urine samples were collected and the acute response (single dosage) study was performed. This day 1 was also the first day of the 7-day study for each subject for each treatment (water or tea, as used in the acute study). The design of the trial is described below.

Acute study (Single dosage):

Baseline fasting venous blood and urine (before the first dose of green tea or hot water) samples were collected. After a single dose of freshly prepared green tea (200ml of 1.5% w/v) or 200ml of hot water (as control), repeat blood samples were taken after 60 and 120 minutes, and urine samples were collected 90 and 180 minutes after consumption. A total of 20ml of venous blood was taken at each time interval, where 2ml were collected into EDTA blood collection tubes and the rest of the blood was collected into heparinised blood collection tubes. Urine samples were collected into clean, dry containers without any preservative for the assessment of polyphenolic profile (performed by another

member of our team, and using LC-MS/MS (Fung *et al*, 2012)). During the sample collection period, i.e. after drinking the single dose of tea or water, subjects were not permitted to eat or drink more than 100ml water. These volunteers were regarded as having taken the first dose of their seven-day supplement.

Seven-day short-term supplementation study (7-day twice daily trial):

From day 1, all subjects were required to drink either 200ml of 1% w/v green tea or hot water twice a day (preferably, once in the morning and once in the evening) for seven consecutive days (tea bags of green tea were supplied), and they returned to the laboratory on day 8, when fasting blood and urine samples were collected. Twenty millilitres fasting venous blood were taken ('post-supplementation') and collected into EDTA (2ml) and heparinised (the rest) tubes. Urine samples were collected into containers without any preservatives.

Subjects then went through a 4-week washout period, after which the procedures of the single dose acute study and 7 days' supplementation were repeated, with each subject crossed-over onto the other treatment. Blood and urine samples were collected again for the acute and seven day study. A total of eight blood and eight urine samples were collected from each subject throughout the whole study period of 8-10 weeks. No diet restriction was imposed upon the subjects, but they were requested not to change their usual diet except for the supplement (green tea/water) only. An outline of this part of the study is shown in Figure 5.1a.

Compliance:

Compliance was assessed by counting up the number of tea bags returned from green tea supplementation group and by inquiry to both groups. A compliance $\geqq$80% was regarded as satisfactory.

Ethical approval:

Ethical approval was obtained from the Human Subjects Ethics Sub-committee of the Hong Kong Polytechnic University.

Power calculations and Statistical analysis:

The key biochemical variable used for power calculation was the Fpg-assisted comet assay results of %DNA in tail. We could find no human hOGG1 activity data or HO-1 expression data for use during the planning of this study. Using the Fpg comet assay data, our previous studies have shown a mean(SD) in healthy subjects of 15.4(2.7)%. With 12 subjects and an SD of 2.7, we will be able to detect a true difference in the mean response of matched pairs of +/-2.4% with 80% power and at the 5% significance level. It was therefore planned to recruit at least 16 subjects to allow for drop-out and/or non-compliance and to maintain a power of 0.8 to detect this level of true difference in the mean response.

Repeated-measures ANOVA with Bonferroni's post-hoc test was performed for the examination of changes in plasma biomarkers, oxidative lesions on lymphocytic DNA (i.e. Fpg comet assay results), hOGG1 activity, and HO-1 protein expression. The paired

Student's t-test was applied to investigate differences in the plasma HO-1 concentration between the day 1 and day 7 samples. An open access gene analysis software tool, Relative Expression Software Tool (REST© 2009, Qiagen), was used to analyse data obtained from real time PCR (Pfaffl *et al*., 2002). This software takes into account the PCR efficiencies of genes of interest and reference genes, and is able to use more than one reference gene for normalisation to increase the reliability of the results. The statistical software uses randomisation and bootstrapping methods to test for the statistical significance of the expression ratios determined. Pearson's correlation was performed to investigate the correlation of variables, including total plasma antioxidant capacity, level of oxidation-induced lesions, hOGG1 activity, and HO-1 protein expression with the plasma polyphenolic content. The data are expressed as mean (SD); $p<0.05$ was considered to be statistically significant.

Preparation of pre-rain Loong-cheng tea for supplementation:

For the acute study, and for those subjects who were randomised to take tea on that occasion, tea bags had been specially prepared by members of our team, each containing 3g of pre-rain Loong-cheng tea leaves (kindly provided by Ying Kee Tea House, HKSAR). A team member (not the investigator) was instructed to prepare the tea for the acute study for each subject by adding one tea bag to 200ml boiling water, with a 3-minute brew time. This tea was regarded as being a 1.5%w/v tea infusion. Just after their fasting samples had been collected, subjects were given the tea, which they drank (under supervision) within 20 minutes. The same volume of boiled water was used as the control treatment. For the supplement used in the 7-day trial, tea bags containing 2g of pre-rain

Loong-cheng tea leaves were prepared and provided to subjects. Subjects were provided with instructions (as provided in **Appendix 3**) on how to prepare tea with the tea bags, and cups provided were marked to a volume of 200ml. The dose was 200ml 1%w/v green tea twice a day, and this was taken for seven days.

Sample collection:

Blood samples were processed immediately after collection. Complete blood count was performed using EDTA-blood, and the remaining EDTA-blood was used for the harvesting of RNA for gene expression experiments. Heparinised blood was used for the other tests. An outline of the sample testing is shown in Figure 5.1b.

The list of the biomarkers investigated is given below,

a) Plasma total bilirubin, triglycerides, high-density lipoprotein (HDL), cholesterol and high-sensitivity C-reactive protein (for hsCRP; only samples collected at baseline and after 7-days' supplementation were examined due to limited resources); total bilirubin was tested immediately due to stability issues; samples for the others were stored at -80^{o}C and batch tested;
b) Total antioxidant capacity of plasma by the FRAP assay was measured immediately after plasma separation;
c) The Comet assays, both Fpg-assisted (to examine the Fpg-labile lesions) and cell extract versions (to assess the activity of the DNA repair enzyme, hOGG1) were performed only on samples collected at baseline and after 7-days'

supplementation); for these, lymphocytes were extracted from whole blood immediately and stored at -80^{o}C for later processing and testing;

d) Gene expression of ARE-related products by real-time PCR used RNA that had been extracted from fresh blood samples and transcribed to cDNA, which was stored at -80^{o}C for later examination. Optimised conditions and primer details are shown in Tables 5.1 and 5.2;

e) Protein expression of ARE related products by Western blotting used lymphocytes that were harvested were stored at -80^{o}C; proteins were extracted before testing. Optimised conditions and antibody details are given in Chapter 3;

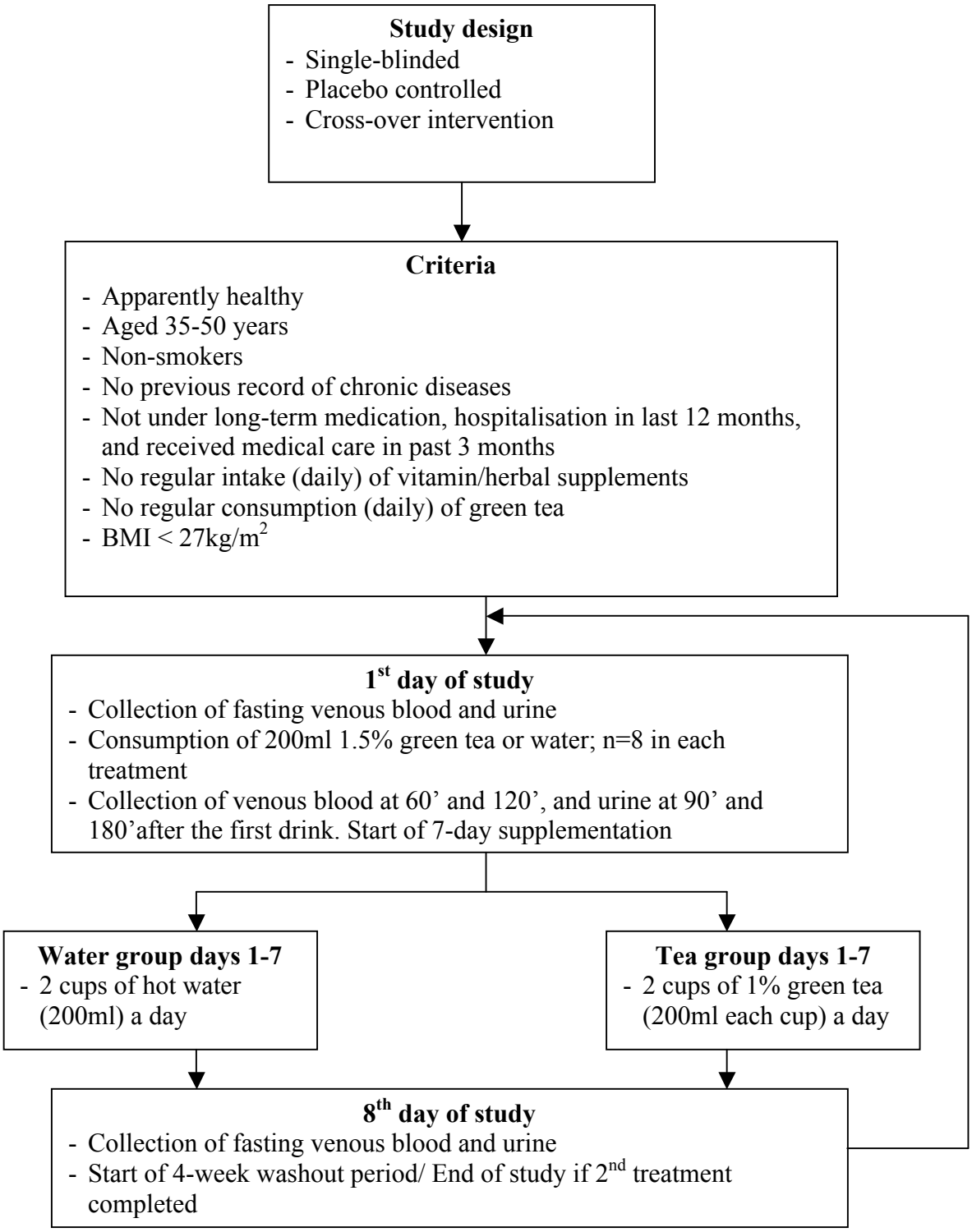
f) Plasma haem oxygenase-1 (HO-1) concentrations by Enzyme-linked immunosorbent assay (ELISA) were measured in heparinised plasma; samples were stored at -80^{o}C for batch testing (only samples collected at baseline and after 7-day supplementation were examined due to limited resources);

g) Polyphenolic content in plasma by LC/MS/MS. This was performed by another member of our group and as described in detail in Fung *et al*, 2012.

The procedures of the above-mentioned tests are described in detail in Chapter 3. It is noted that all stored samples from the same subject were run in parallel in the same batch in order to minimise experimental variation.

Figure 5.1 Outline of this part of the study. (a) Experimental design and flow; (b) Tests to be done on samples collected

(a) Experimental design and flow.



(b) Summary of tests done on samples collected

	Tests	Baseline	Post 60'	Post 120'	7-day
Heparinised Plasma (from heparinised blood)	FRAP	✓	✓	✓	✓
	Cholesterol	✓	✓	✓	✓
	HDL	✓	✓	✓	✓
	Triglycerides	✓	✓	✓	✓
	hsCRP	✓	Nil	Nil	✓
	Polyphenolic content	✓	✓	✓	✓
Lymphocytes (from heparinised blood)	Fpg lesions	✓	✓	✓	✓
	hOGG1 activity	✓	Nil	Nil	✓
	Protein expression	✓	✓	✓	✓
RNA (from EDTA blood)	Gene expression	✓	✓	✓	✓

Note: Tests were done on samples from both tea and water treatments. For day 1 of tea treatment, 200ml 1.5% green tea was provided to subjects and the following doses until day 7 were 200ml 1% green tea (twice a day). The first dose was higher in concentration so as to trigger an initial response in acute study, and the following lower doses for maintenance.

Table 5.1. Preparation of master mix for real time PCR

a) Composition of master mix for a ***single*** reaction.

Reagents	Volume (µl)	Final concentration
10X PCR Buffer	1.0	/
2mM dNTP	1.0	0.1mM
10µM Forward primer	0.3	300nM
10µM Reverse primer	0.3	300nM
25mM $MgCl_2$	Primer dependent	Primer dependent
10X SYBR® Green*	0.1	0.1X
5µM ROX®	0.1	50nM
MilliQ water	Make up to 9µl	/
5U/µl HotStarTaq® DNA Polymerase	0.08	0.4U

*10X SYBR® Green was prepared *in situ* from dilution of 100X stock SYBR® Green.

b) $MgCl_2$ concentrations used for each pair of primers:

[$MgCl_2$] (mM)	Primer used	Volume added (µl)/ Rx
2	*NQO1*	0.8
2.5	*CD8β, KEAP1, XRCC5*	1.0
3	*CD3ε, HMOX1, NRF2, BACH1, hOGG1, GSTα*	1.2

Table 5.2. Primers for each gene of interest

Gene	Orientation	Sequence	Size (bp)
CD3ε	Forward	5′-GGCAGGCAAAGGGGACA-3′	236
	Reverse	5′-ACCATGAGGCTGAGGAACGAT-3′	
CD8β	Forward	5′-GACAGTCACCACGAGTTCCT-3′	133
	Reverse	5′-GCTTCACGCTTGTGAGATTGAGAA-3′	
HMOX1	Forward	5′-CAGGCAGAGAATGCTGAGTTC-3′	146
	Reverse	5′-GATGTTGAGCAGGAACGCAGT-3′	
NRF2	Forward	5′-AGTGGATCTGCCAACTACTC-3′	106
	Reverse	5′-CATCTACAAACGGGAATGTCTG-3′	
KEAP1	Forward	5'-ATGCCTCAGTGTTAAAATGACAT-3'	224
	Reverse	5'-CAGGTATCCAAGAATAAATCACA-3'	
BACH1	Forward	5′-TGCGATGTCACCATCTTTGT-3′	100
	Reverse	5′-CCTGGCCTACGATTCTTGAG-3′	
hOGG1	Forward	5′-ACACTGGAGTGGTGTACTAGCG-3′	301
	Reverse	5′-GCCGATGTTGTTGTTGGAGG-3′	
XRCC5	Forward	5′-GCGACAGGTGTTTGCTGAGA-3′	78
	Reverse	5′-GAAAGGGGATTGTCAGTGCCAT-3′	
NQO1	Forward	5′-GAGGTACAGGATGAGGAGAAAAA-3′	84
	Reverse	5′-CTGGTTGTCAGTTGGGATGGA-3′	
GSTα	Forward	5′-GGCTGACATTCATCTGGTGG-3′	199
	Reverse	5′-CCTGAAAATCTTCCTTGCTTCTTCT-3′	

Results:

Concentrations of plasma biomarkers, including total bilirubin, triglycerides, high-density lipoprotein (HDL), cholesterol and the total antioxidant capacity (FRAP) in plasma

Plasma cholesterol, high-density cholesterol (HDL-C), triglycerides, uric acid, and total bilirubin were examined in the acute and 7-day studies. No significant differences ($p>0.05$) were observed between the two treatments (tea and water) groups (Table 5.3b). Plasma hsCRP concentration was only examined in samples collected at baseline and after 7-day supplementation, and no significant change was observed. The total antioxidant capacity, measured as FRAP, after drinking green tea was found to be increased at 60' and 120', but the difference did not reach statistical significance in this acute study (Tables 5.3a & 5.3b).

Table 5.3. Biomarker results examined from heparinised plasma collected at baseline, 60min and 120min after drinking green tea and after 7 days' supplementation with green tea or water. (a) Values of biomarkers as measured in acute and 7-day studies and presented in mean(SD) and range; (b) Differences of each biomarker compared to corresponding baseline concentrations in acute and 7-day studies. Results are presented as mean(SD) of 16 subjects

(a) Values of biomarkers as measured in acute and 7-day studies and presented in mean(SD) and range

	Baseline		Post 60'		Post 120'		7-day	
	Tea n=16	Water n=16	Tea n=16	Water n=15	Tea n=16	Water n=16	Tea n=16	Water n=16
Cholesterol (mmol/l)	4.1(0.6); 3.4-5.7	4.1(0.4); 3.5-4.7	4.2(0.6); 3.5-5.5	4.1(0.4); 3.5-4.7	4.1(0.6); 3.5-5.6	4.1(0.4); 3.6-4.8	4.1(0.5); 3.3-5.5	4.1(0.5); 3.2-4.9
HDL (mmol/l)	1.2(0.9); 0.6-1.6	1.2(0.3); 0.7-1.6	1.3(0.3); 0.7-1.6	1.2(0.3); 0.7-1.6	1.3(0.3); 0.7-1.6	1.2(0.3) 0.7-1.6	1.2(0.3); 0.7-1.6	1.2(0.3); 0.6-1.7
LDL (mmol/l)	2.5(0.6); 1.8-4.0	2.5(0.4); 1.9-3.1	2.5(0.5); 1.8-3.9	2.5(0.4); 1.8-3.3	2.5(0.6); 1.8-4.0	2.5(0.4); 1.8-3.2	2.5(0.5); 1.9-3.9	2.4(0.5); 1.4-3.4
Triglycerides (mmol/l)	0.9(0.7); 0.4-3.0	0.9(0.6); 0.4-2.6	0.9(0.7); 0.5-3.0	0.9(0.5); 0.5-2.4	0.9(0.7); 0.4-3.0	0.9(0.5); 0.4-2.4	0.9(0.8); 0.4-3.3	1.1(0.9); 0.5-4.2
Uric acid (μmol/l)	271(54); 173-369	271(57); 181-358	272(52); 181-376	270(60); 185-361	269(52); 177-366	265(56); 181-349	275(59); 176-388	276(53); 181-361
Total bilirubin (μmol/l)	18.1(10.0); 6.1-47.2	17.3(10.5); 8.4-50.1	19.1(11.0); 6.9-53.1	17.8(12.3); 8.5-56.7	19.9(11.9); 6.9-57.7	18.5(13.1) 8.5-62.6	15.5(11.6); 6.0-55.3	15.1(7.8); 3.6-38.1
hsCRP (nmol/l)	2.0(1.1); 0.7-5.2	1.9(1.1); 0.6-4.9	N/A	N/A	N/A	N/A	2.0-1.2; 0.5-4.6	2.7(2.0); 0.7-7.2
FRAP (μmol/l)	858(205); 559-1197	835(190); 549-1215	882(211); 583-1241	840(172); 569-1200	881(196); 580-1214	848(167); 575-1181	853(210); 525-1300	870(253); 540-1225

(b) Differences of each biomarker compared to corresponding baseline concentrations in acute and 7-day studies

	Post 60' - Baseline		Post 120'- Baseline		7-day- Baseline		
	Tea n=16	Water n=15	Tea n=16	Water n=16	Tea n=16	Water n=16	p-value
Cholesterol (mmol/l)	0.1(0.1)	0.0(0.1)	0.0(0.1)	0.0(0.1)	0.0(0.2)	0.0(0.3)	n.s.
HDL (mmol/l)	0	0.0(0.1)	0	0	0.0(0.1)	0.0(0.1)	n.s.
LDL (mmol/l)	0.0(0.1)	0.0(0.1)	0.0(0.1)	0.0(0.1)	0.0(0.2)	0.0(0.4)	n.s.
Triglycerides (mmol/l)	0.0(0.1)	0.0(0.1)	0.0(0.1)	0.0(0.1)	0.0(0.2)	0.2(0.5)	n.s.
Uric acid (µmol/l)	1.4(6.3)	-2.2(6.8)	-2.1(8.4)	-6.3(8.3)	4.8(29.3)	5.5(21.5)	n.s.
Total bilirubin (µmol/l)	1.0(1.9)	0.5(2.7)	1.8(2.9)	1.3(3.7)	-2.6(6.3)	-2.2(5.9)	n.s.
hsCRP (nmol/l)	N/A	N/A	N/A	N/A	-0.2(1.6)	0.8(2.0)	n.s.
FRAP (µmol/l)	24.3(76.3)	1.4(42.6)	23.5(80.9)	13.0(43.7)	-4.7(219.1)	25.0(239.3)	n.s.

Oxidative DNA damage in human lymphocytes

As can be seen in Tables 5.3 & 5.4 and Figures 5.2 & 5.3, significantly lower DNA damage ($p<0.001$) was observed after drinking green tea (11.62±2.45%; -5.22±2.23%), 120' (11.70±2.28%; -5.13±2.47%) and one week (11.62±1.82%; -5.22±3.10%) from the baseline level of 16.84±3.09% (Figure 5.2), but not after drinking water and significant differences were found at all time points between tea and water treatments (Figure 5.3; Individual data are also shown in Table 5.4). The level of Fpg lesions were found to be decreased ~31% at 60' and 120' after the first cup of green tea, and the extent of decrease was maintained during the one-week green tea supplementation (Figure 5.3 & Table 5.5).

Table 5.4. Results (as %DNA in comet tail using the Fpg-assisted comet assay) before and 60min and 120min in the acute study, and in the fasting samples collected after 7 days of regular consumption. Results are presented as mean and SD from 16 subjects

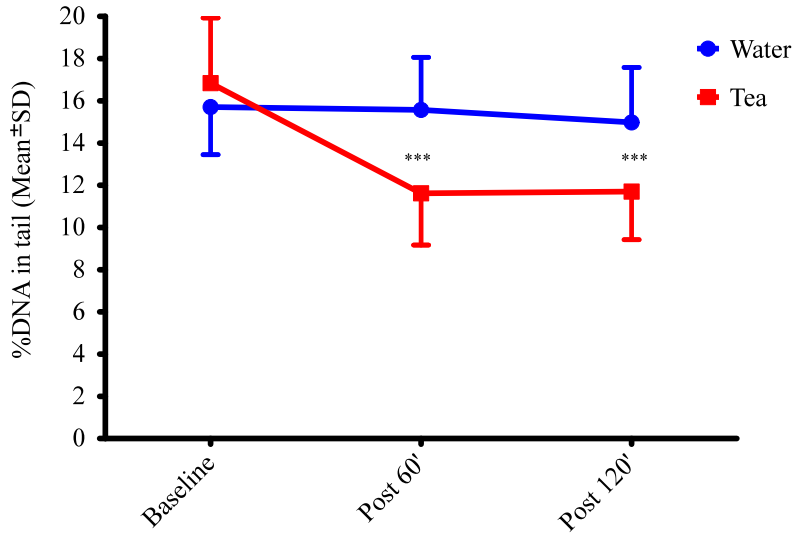
	Water				Green tea			
	Baseline	**Post 60'**	**Post 120'**	**7-day**	**Baseline**	**Post 60'**	**Post 120'**	**7-day**
ASG01	17.92	14.80	13.32	13.69	14.37	11.89	13.85	15.82
ASG02	14.02	14.01	14.39	19.66	15.18	11.47	11.50	11.61
ASG03	15.51	13.08	12.87	12.13	17.42	10.67	14.00	12.97
ASG04	14.29	13.85	12.62	13.05	12.34	10.05	9.00	10.15
ASG05	17.29	17.03	14.53	14.88	21.99	15.43	14.32	13.24
ASG06	18.77	17.94	18.05	18.91	16.49	10.28	11.79	9.91
ASG07	16.27	14.90	16.26	15.79	16.53	8.57	9.81	10.23
ASG08	13.78	12.74	12.87	12.27	13.84	7.95	8.97	9.40
ASG09	19.09	18.14	20.66	22.43	17.53	14.04	15.04	13.45
ASG10	16.07	17.25	16.40	16.96	16.42	12.54	10.80	12.38
ASG11	11.88	13.69	11.36	12.30	23.36	13.42	13.02	10.57
ASG12	13.06	13.85	14.23	13.18	14.27	12.15	10.71	10.82
ASG13	12.82		12.01	12.17	13.67	9.04	8.43	9.39
ASG14	18.39	21.60	18.84	18.37	20.01	15.53	15.32	12.67
ASG15	15.14	13.55	15.21	14.39	16.25	8.65	9.93	10.20
ASG16	17.04	17.07	16.05	20.23	19.69	14.17	10.75	13.07
	Baseline	**Post 60'**	**Post 120'**	**7-day**	**Baseline**	**Post 60'**	**Post 120'**	**7-day**
Mean	15.71	15.57	14.98	15.69	16.84	11.62	11.70	11.62
SD	2.26	2.49	2.60	3.34	3.09	2.45	2.28	1.82

Table 5.5. Difference in DNA damage (as %DNA in comet tail using the Fpg-assisted comet assay) after tea and water in the acute and 7-day trial. Results are presented as mean and SD from 16 subjects

	Water			Green tea		
	Δ Post 60' from Baseline	**Δ Post 120' from Baseline**	**Δ 7-day from Baseline**	**Δ Post 60' from Baseline**	**Δ Post 120' from Baseline**	**Δ 7-day from Baseline**
ASG01	-3.12	-4.60	-4.23	-2.48	-0.52	1.45
ASG02	-0.01	0.37	5.64	-3.71	-3.68	-3.57
ASG03	-2.43	-2.64	-3.38	-6.75	-3.42	-4.45
ASG04	-0.44	-1.67	-1.24	-2.29	-3.34	-2.19
ASG05	-0.26	-2.76	-2.41	-6.56	-7.67	-8.75
ASG06	-0.83	-0.72	0.14	-6.21	-4.70	-6.58
ASG07	-1.37	-0.01	-0.48	-7.95	-6.72	-6.30
ASG08	-1.05	-0.92	-1.52	-5.90	-4.87	-4.44
ASG09	-0.95	1.57	3.34	-3.49	-2.48	-4.08
ASG10	1.18	0.33	0.89	-3.88	-5.62	-4.04
ASG11	1.81	-0.52	0.42	-9.94	-10.34	-12.79
ASG12	0.79	1.18	0.12	-2.12	-3.57	-3.45
ASG13		-0.81	-0.65	-4.63	-5.24	-4.28
ASG14	3.21	0.45	-0.02	-4.48	-4.69	-7.34
ASG15	-1.59	0.07	-0.15	-7.60	-6.32	-6.05
ASG16	0.03	-0.99	3.19	-5.52	-8.94	-6.62
	Δ Post 60' from Baseline	**Δ Post 120' from Baseline**	**Δ 7-day from Baseline**	**Δ Post 60' from Baseline**	**Δ Post 120' from Baseline**	**Δ 7-day from Baseline**
Mean	-0.34	-0.73	-0.02	-5.22	-5.13	-5.22
SD	1.62	1.58	2.50	2.23	2.47	3.10

Figure 5.2 Effects of drinking green tea and water (a) 60min and 120min after drinking the first cup of green tea and (b) for 7 days on the oxidation-induced lesions in terms of % DNA in tail after Fpg-treatment. Results are presented as mean±SD (n=16) and *** represents $p<0.001$ in comparison to baseline

(a)



(b)

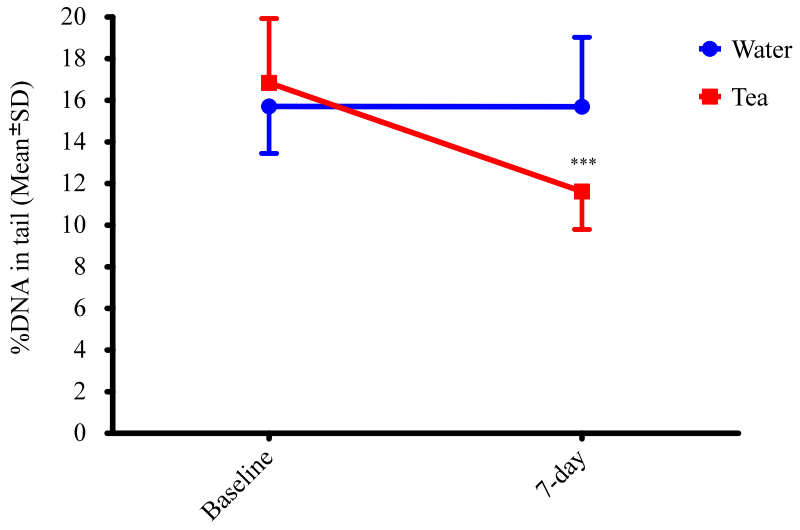
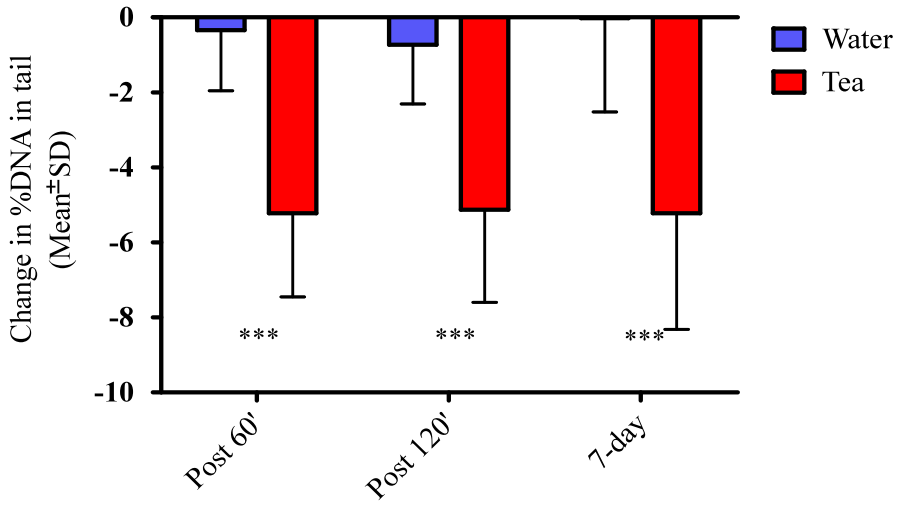


Figure 5.3 Effects of drinking green tea and water 60min and 120min after drinking the first cup of green tea and for 7 days on the oxidation-induced lesions in terms of %DNA in tail after Fpg-treatment. Results are presented as mean±SD (n=16); and *** represents $p<0.001$ in comparison to water treatment.



Activity of DNA base excision repair enzyme, hOGG1, in human lymphocytes before and after green tea supplementation (change in DNA repair enzyme activity)

The version of the comet assay used to measure the activity of hOGG1 requires the use of a photosensitiser (PS), Ro19-8022, to induce oxidative lesions in a controlled manner in substrate cells, (HeLa cells were used in this study). These lesions are recognised and changed to single strand breaks by hOGG1 in the cell extracts made from the subjects' lymphocytes. Preliminary testing to confirm the introduction of oxidative lesions by Ro19-8022 in the substrate cells, under four conditions (and as revealed by Fpg and by a cell extract made for this preliminary testing) was performed (please refer to Chapter 3 for details of the methodology). Results are shown in Figure 5.4. A low background level of oxidative lesions was observed in HeLa cells without PS and light exposure, and the addition of either light or PS did not introduce additional oxidative lesions to the HeLa cells. However, under the exposure of both PS and light, a marked increase in oxidative lesions was observed in Fpg-treated HeLa cells, and this was also found in the HeLa cells treated with hOGG1 extracted from pooled lymphocytes with the same number of cells used as real samples (Figure 5.4). The %DNA scores in the photosensitised damaged HeLa cells treated with Fpg- or cell extract (containing hOGG1) were both around 19%. The same batch of substrate cells was used in subsequent experiments for determination of hOGG1 activity using cell extracts from the test subjects.

Due to some low volume blood samples, only 13 complete sets of cell extract samples were available. As can be seen in Figure 5.5, no significant difference was observed in baseline levels of the %DNA in tail of the extract-treated cells prior to tea (11.86±3.42%)

and water (12.71±2.83%) treatments. Drinking water for 7 days did not result in a significant change in %DNA in the tail of extract treated substrate cells (11.65±3.45% after 7 days). However, the %DNA in the tail of extract-treated cells was found to be increased significantly (to 16.7±4.08% after 7 days; p=0.0006) with seven days regular tea drinking, indicating increased hOGG1 activity with green tea (Figure 5.5). There was a significant difference (p<0.001) between the day 1-7 change in %DNA in tail of extract-treated substrate cells with water (-1.06±1.63%) and green tea (+4.84±3.35%) (shown in Figure 5.6).

Figure 5.4 The %DNA in comet tail in HeLa cells treated under four conditions: (A) no PS and no light; (B) no PS but light; (C) with PS but no light; (D) with both PS and light, after Fpg treatment, in particular condition D was tested with either Fpg or extract from pooled lymphocytes. Results are presented as mean±SD of 2 gels/ treatment with 50 cells scored per gel per experiment and with three independent experiments performed (*** represents p<0.001).

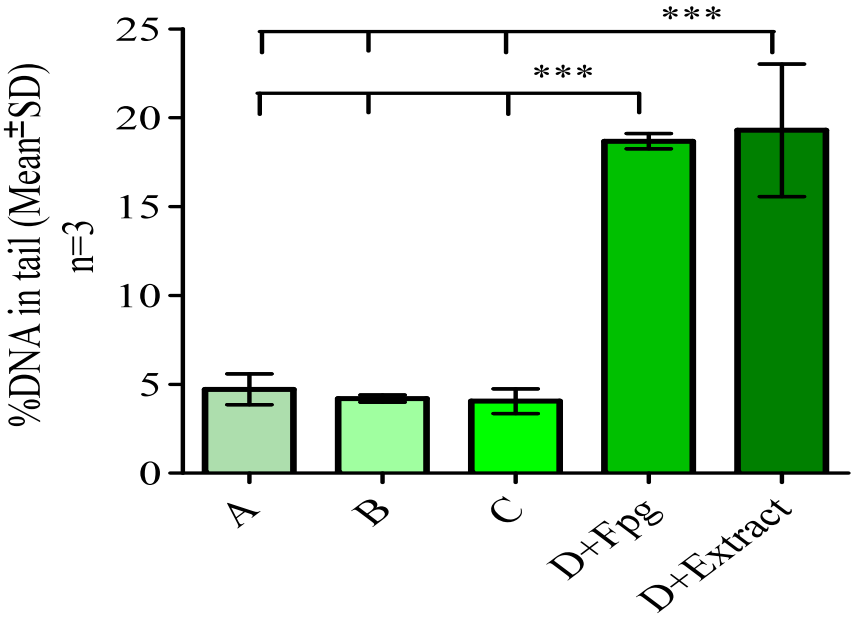


Figure 5.5 Effects of 7-days' green tea supplementation on the activity of hOGG1, assessed using the cell extract comet assay with substrate cells. Results are presented as mean±SD from 13 subjects and *** represents p<0.001

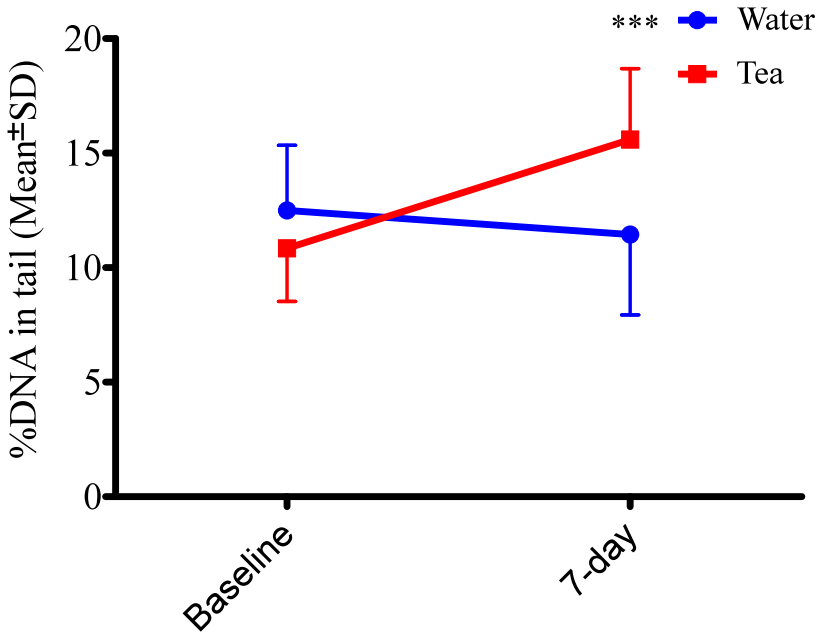
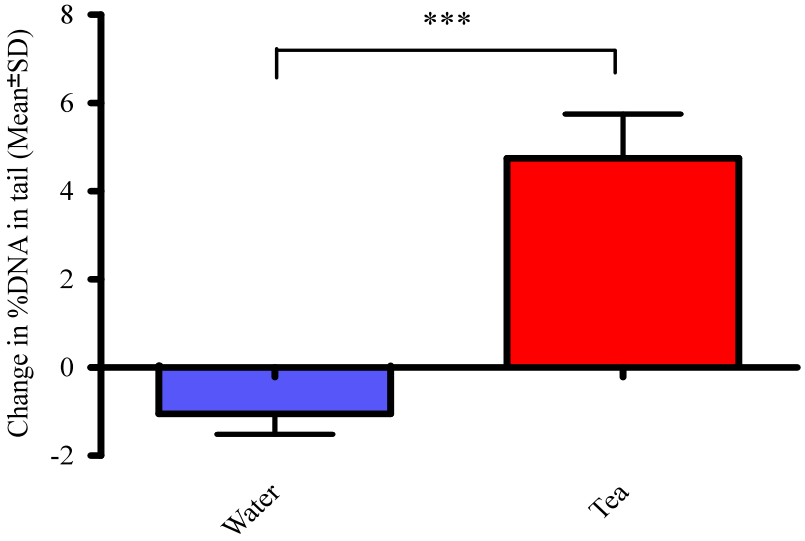


Figure 5.6 The day 1-7 change in activity of hOGG1, assessed using the cell extract comet assay with substrate cells. Results are presented as mean±SD from 13 subjects and *** represents p<0.001



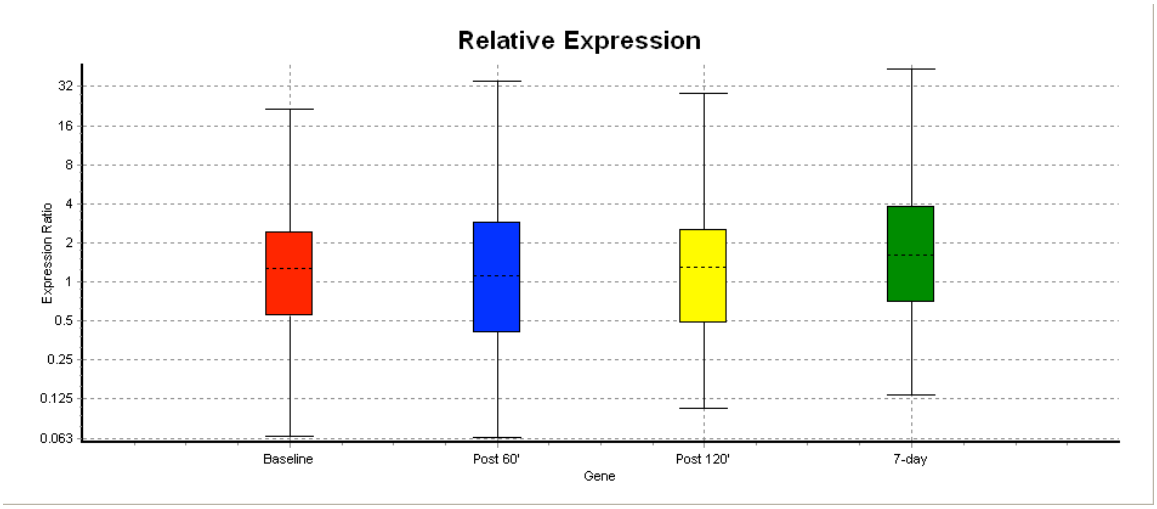
Gene expression of ARE-related products:

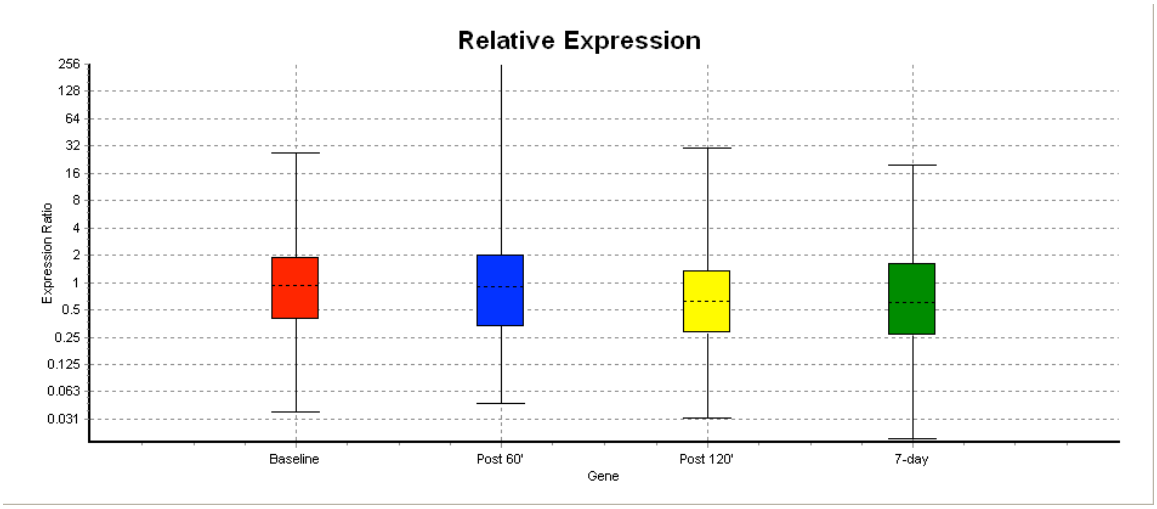
Several genes of interest were studied. They were *HMOX1*, *NRF2*, *KEAP1*, *BACH1*, *NQO1*, *GSTα*, *XRCC5* and *hOGG1*. As in Chapter 4, two reference genes, *CD3ε* and *CD8β* were applied to normalise the results obtained. REST© 2009 was used for statistical analysis where randomisation and bootstrapping methods were applied to test for the statistical significance ($p<0.05$) of the expression ratios determined.

Among the genes studied (*HMOX1*, *NRF2*, *KEAP1*, *BACH1*, *NQO1*, *GSTα*, *XRCC5* and *hOGG1,* with two reference genes, *CD3ε* and *CD8β*, as used in the work described in Chapter 4), no significant differences were observed as a result of tea in either the acute study or after 7-day consumption. The baseline level of each gene prior to drinking tea or water was not significantly different. With regard to *hOGG1*, even though hOGG1 activity was seen to be increased (as described in the previous section and shown in Figures 5.5 and 5.6), no change in gene expression was observed.

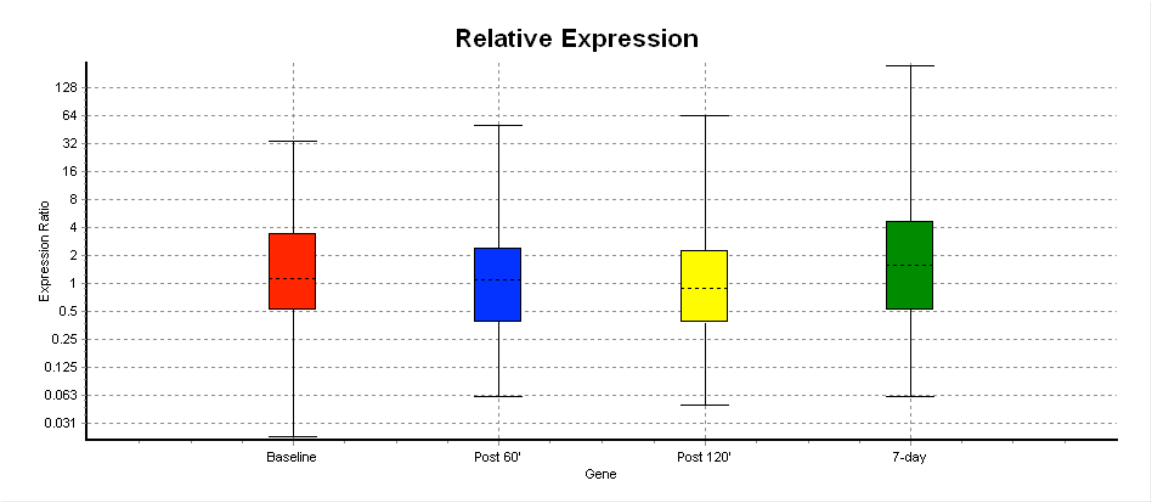
Figure 5.7 Effects of tea supplementation on ARE-related genes in lymphocytes, (a) *HMOX1*, (b) *NRF2*, (c) *KEAP1*, (d) *BACH1*, (e) *hOGG1*, (f) *NQO1*, (g) *GSTα*, (h) *XRCC5*, using both *CD3ε* and *CD8β* as reference genes, where the upper figure of each gene shows the effect of tea supplementation and the lower the effect of water control. Data are presented using box and whisker plots, with whiskers showing the range, the box represents the lower and upper quartiles, and the dotted line in the box representing the median.

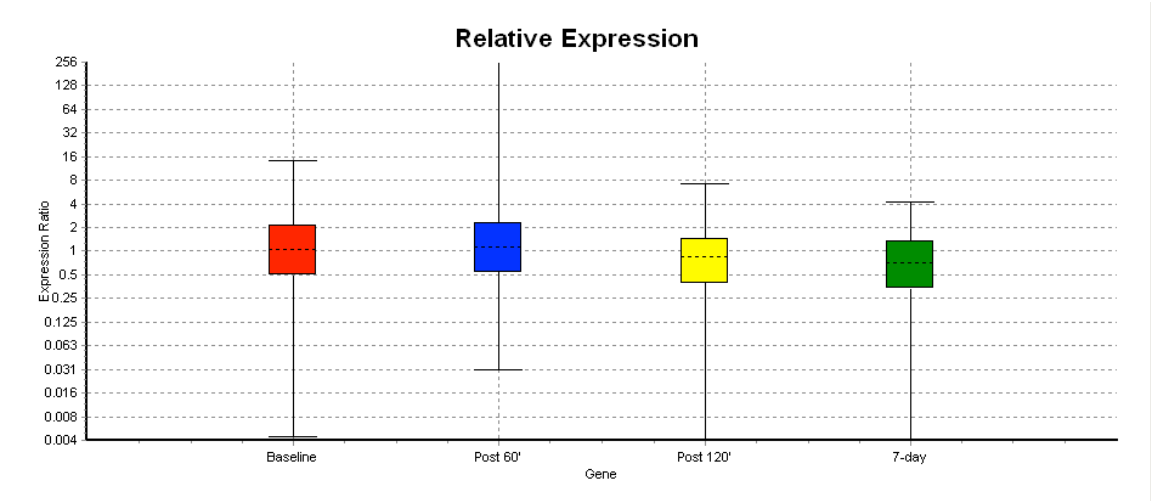
(a) HMOX1





(b) NRF2


Relative Expression
Expression Ratio
128
64
32
16
8
4
2
1
0.5
0.25
0.125
0.063
0.031
Baseline
Post 60'
Post 120'
7-day
Gene


Relative Expression
Expression Ratio
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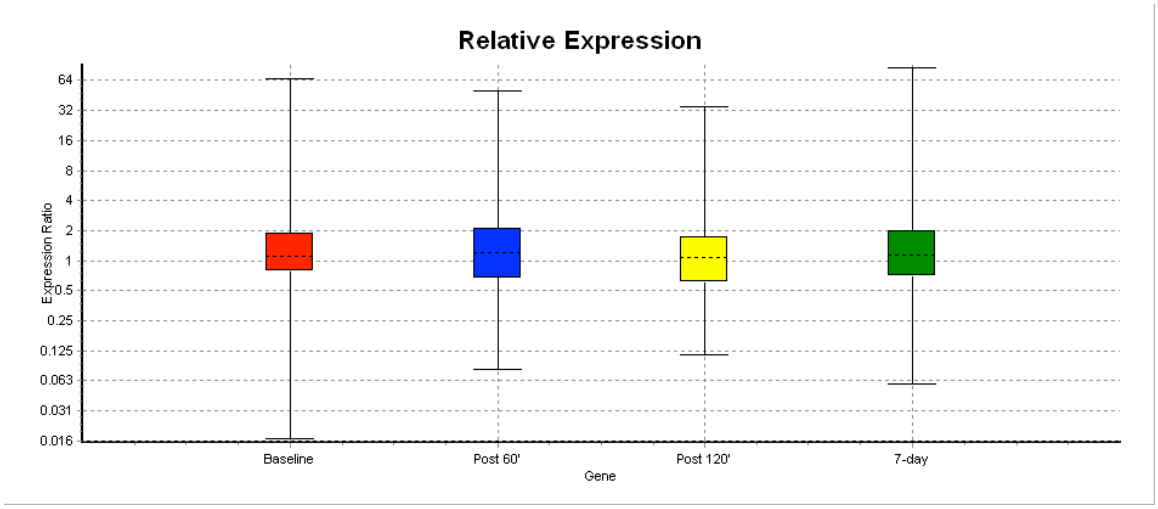
128
64
32
16
8
4
2
1
0.5
0.25
0.125
0.063
0.031
0.016
0.008
0.004
Baseline
Post 60'
Post 120'
7-day
Gene

(c) KEAP1


Relative Expression
Expression Ratio
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32
16
8
4
2
1
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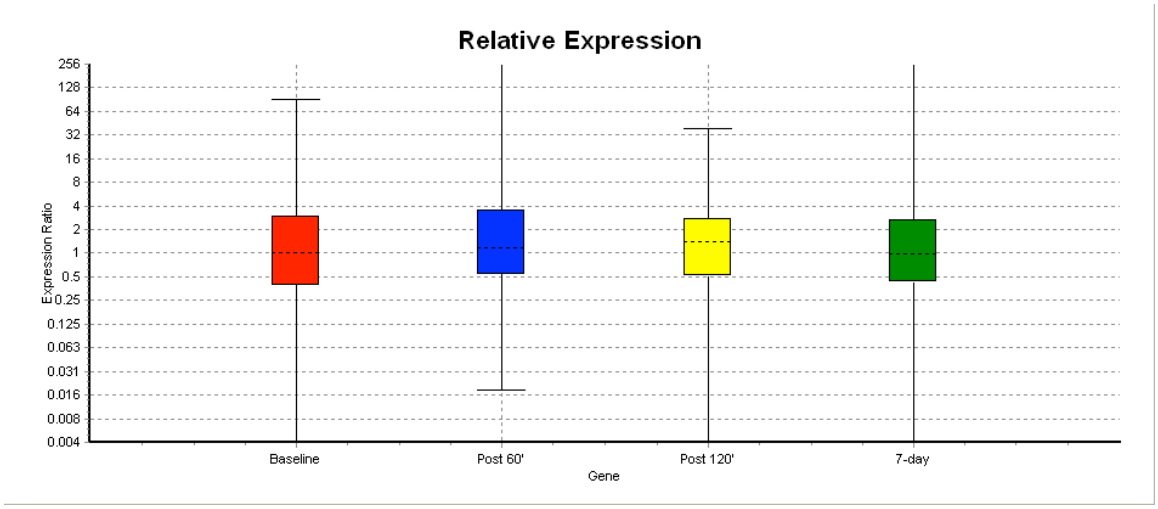
0.016
Baseline
Post 60'
Post 120'
7-day
Gene


Relative Expression
Expression Ratio
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64
32
16
8
4
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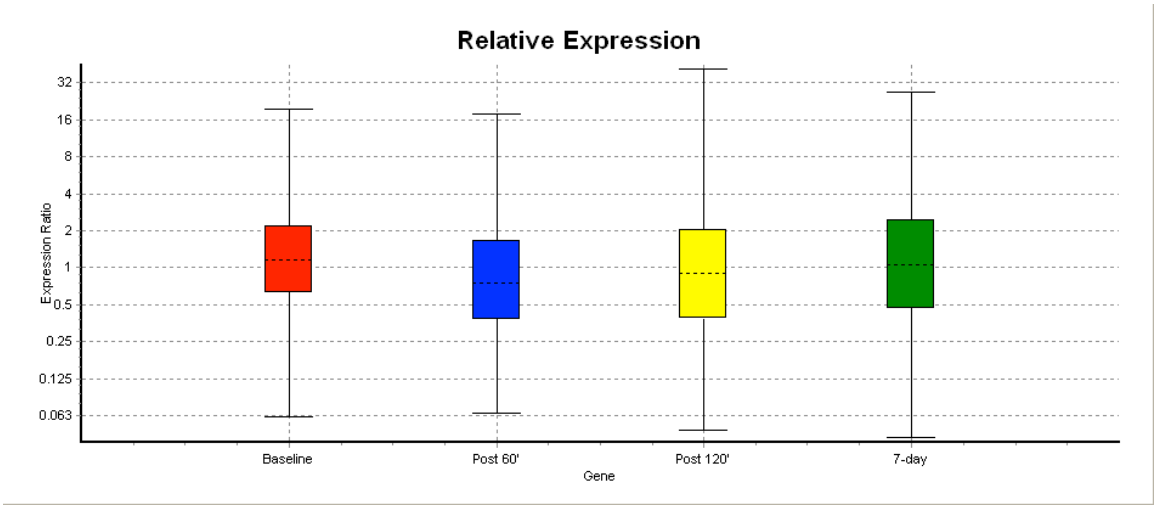
1
0.5
0.25
0.125
0.063
0.031
0.016
0.008
0.004
Baseline
Post 60'
Post 120'
7-day
Gene

(d) BACH1


Relative Expression
Expression Ratio
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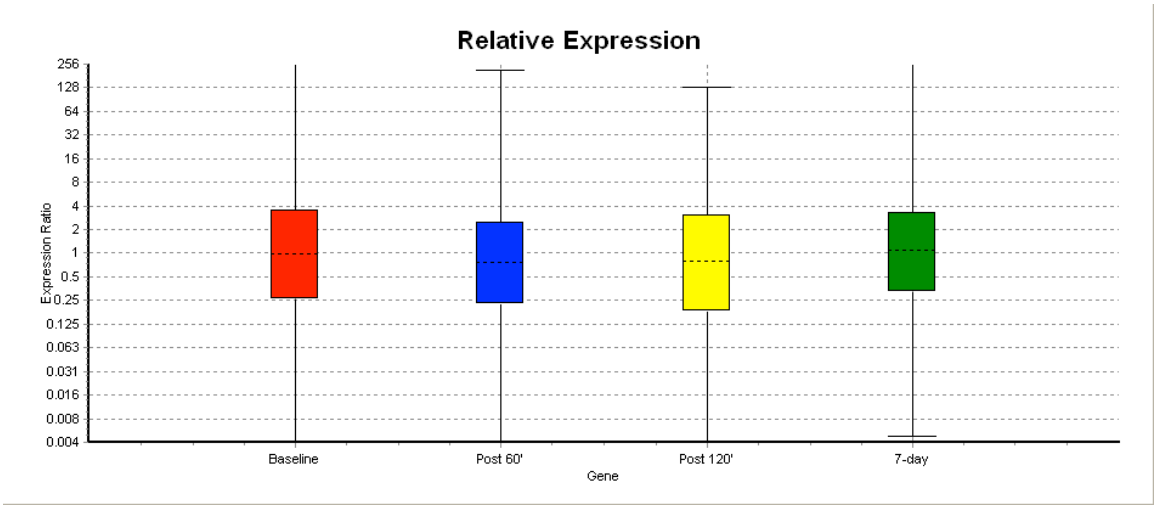
16
8
4
2
1
0.5
0.25
0.125
0.063
Baseline
Post 60'
Post 120'
7-day
Gene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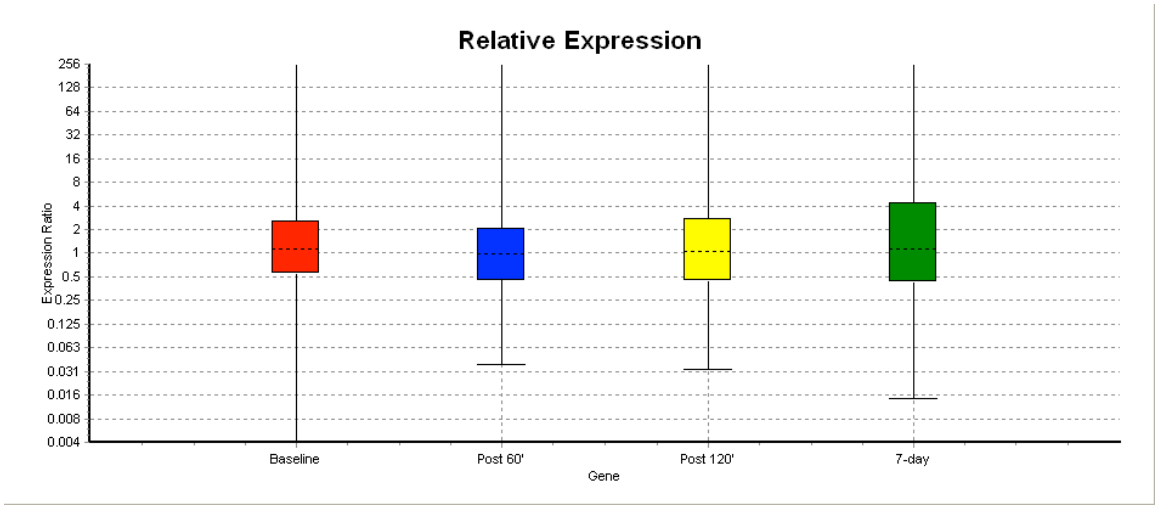
0.008
0.004
Baseline
Post 60'
Post 120'
7-day
Gene

(e) hOGG1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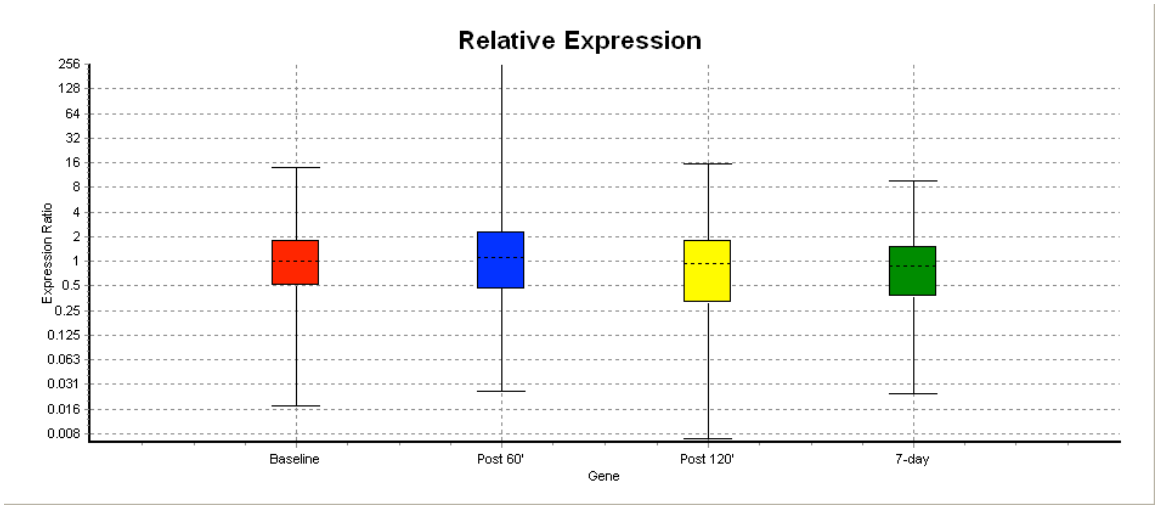
0.004
Baseline
Post 60'
Post 120'
7-day
Gene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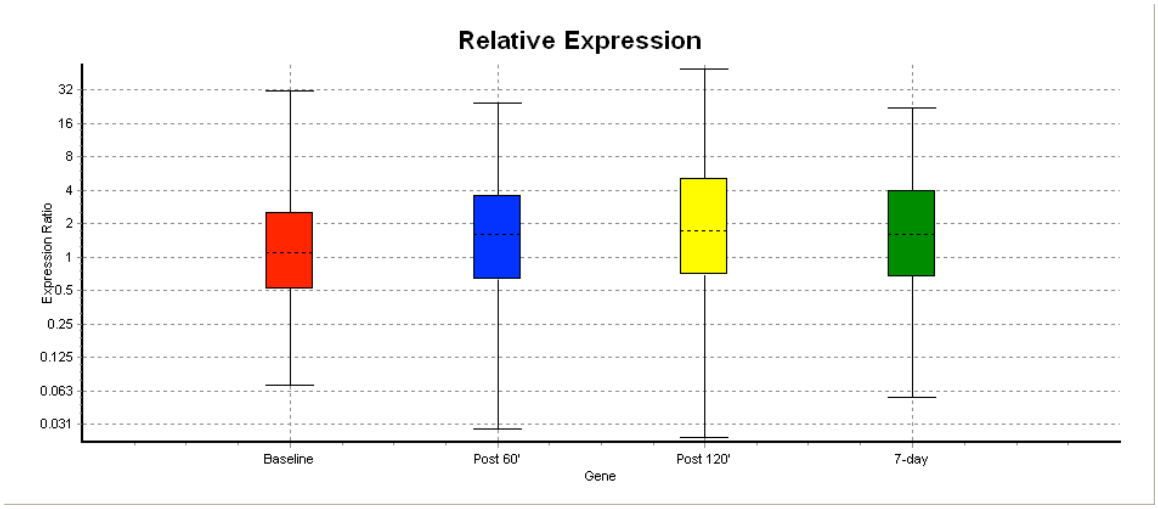
0.008
Baseline
Post 60'
Post 120'
7-day
Gene

(f) NQO1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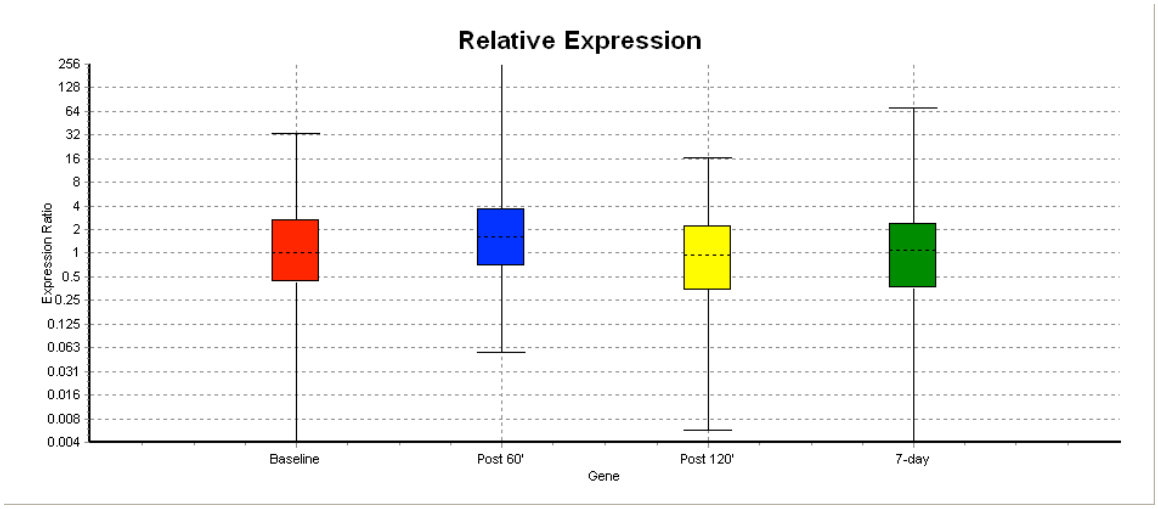
Gene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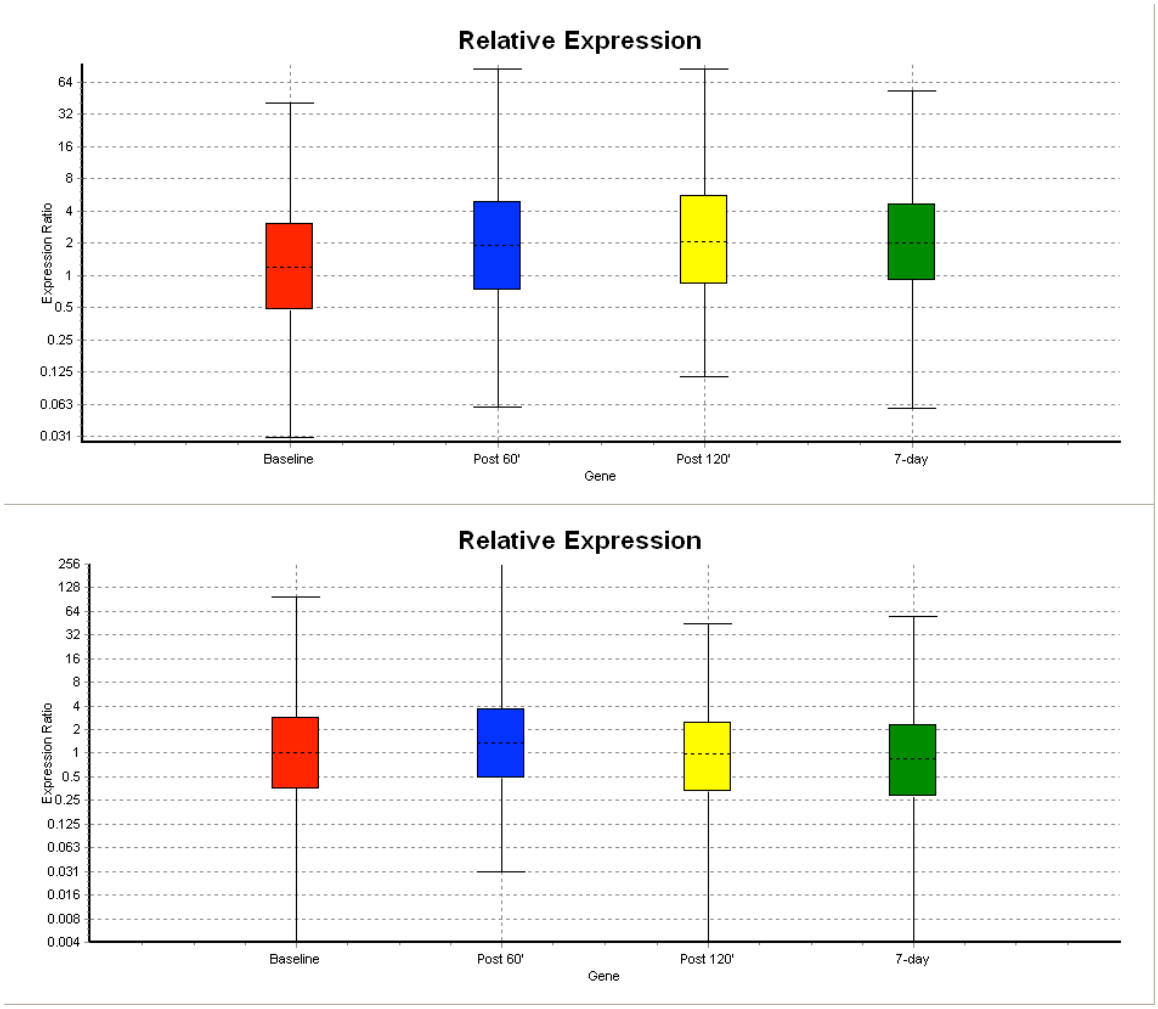
0.008
0.004
Baseline
Post 60'
Post 120'
7-day
Gene

(g) GSTα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Post 120'
7-day
Gene
Relative Expression
Expression Ratio
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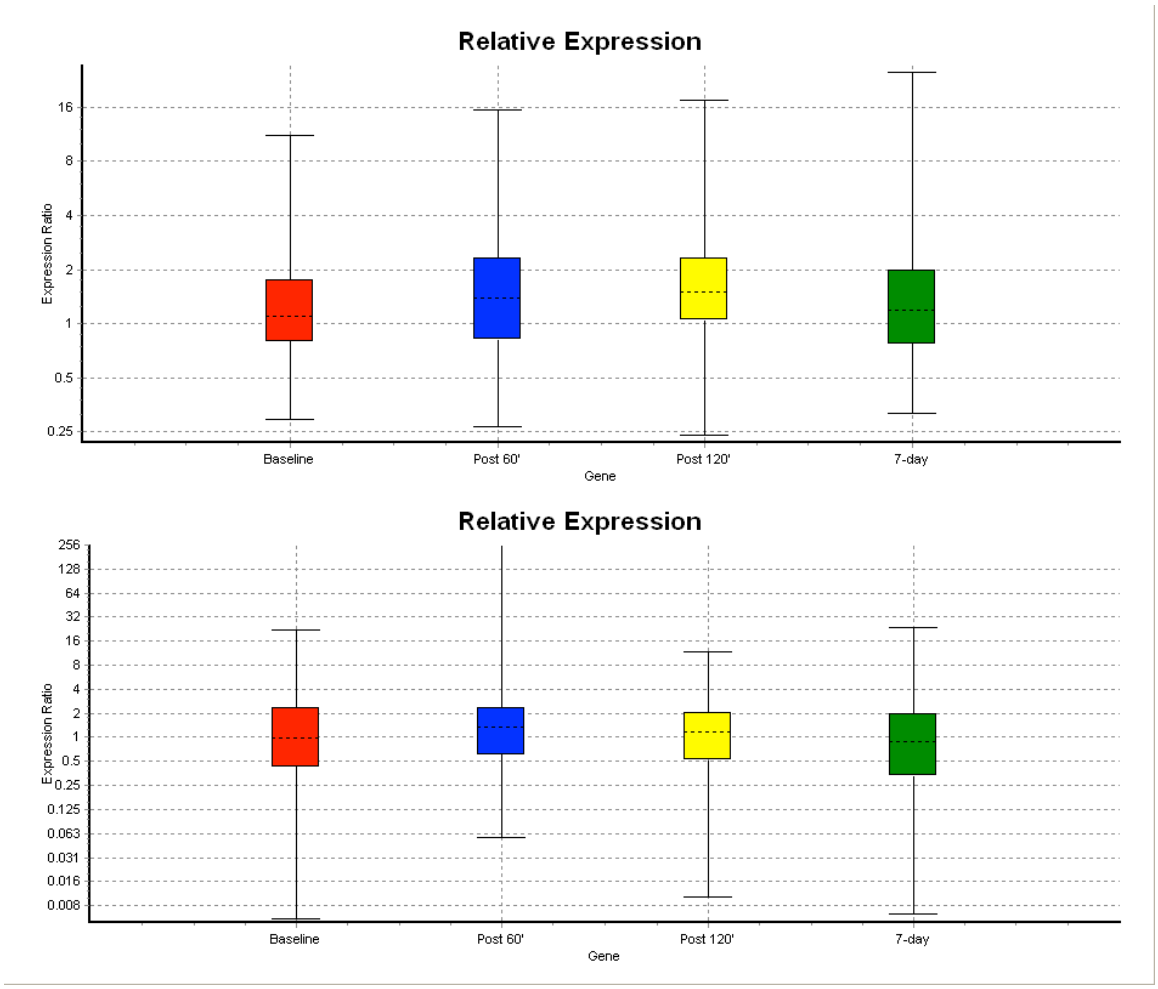
Baseline
Post 60'
Post 120'
7-day
Gene

(h) XRCC5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7-day
Gene
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4
2
1
0.5
0.25
0.125
0.063
0.031
0.016
0.008
Baseline
Post 60'
Post 120'
7-day
Gene

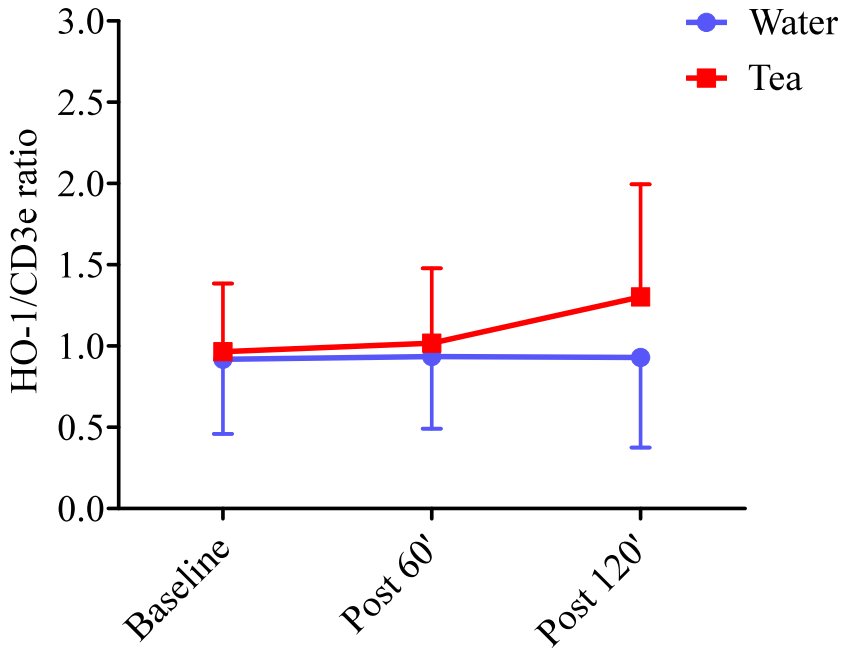
Protein expression of ARE-related products

CD3ε was used to normalise the results obtained. Carestream Molecular Imaging (New Haven, CT, UK) was used to quantify the protein concentrations.

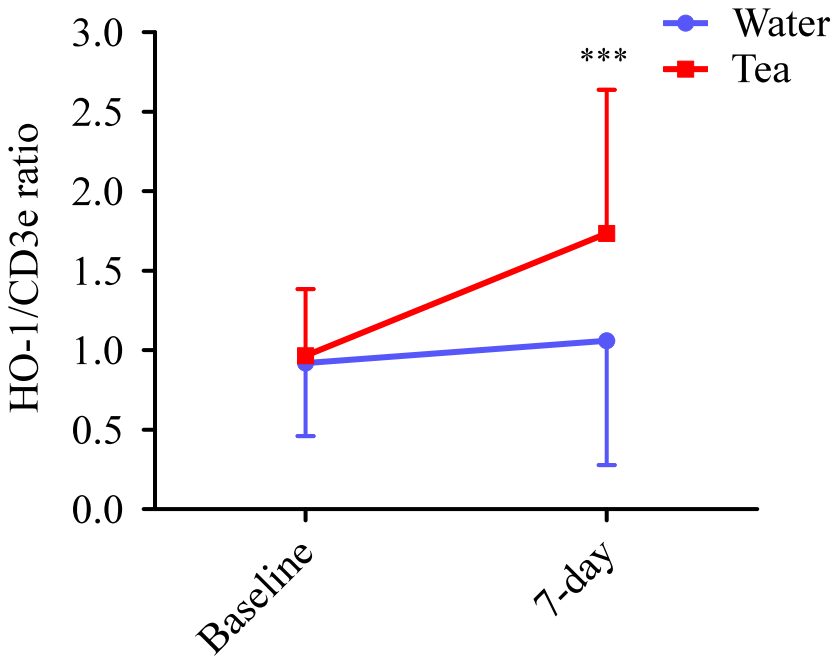
HO-1 protein expression was not found to be significantly different at 60 minutes (1.02±0.46) or 120 minutes (1.30±0.69) after drinking tea compared to drinking water (60 minutes: 0.94±0.44; 120 minutes: 0.93±0.55), although a small increase in expression of HO-1 was seen 120 minutes after drinking green tea (Figure 5.8a). In the seven-day trial, a significant increase ($p=0.0035$) in HO-1 expression was seen, from 0.97±0.42 (baseline) to 1.74±0.90 (7-day), compared to water (baseline level: 0.92±0.46; 7-day level: 1.06±0.78; Figure 5.8b).

Figure 5.8 Effects of drinking green tea and water on protein expression of haem oxygenase-1 (HO-1): (a) 60min and 120min after drinking the first cup of green tea (day 1) and (b) after 7 days regular intake of green tea. Results are presented as mean±SD from results of 16 subjects and *** represents p<0.001

(a)



(b)



Plasma haem oxygenase-1 concentration before and after green tea supplementation

Due to limited resources, only samples collected at baseline and after 7-day supplementation were examined. In Figure 5.9, it can be seen that there was no significant difference between the baseline and post-7-day concentrations of plasma HO-1 between tea (baseline level: 1.34±0.46ng/ml; 7-day: 1.31±0.49ng/ml) and water (baseline level: 1.38±0.54ng/ml; 7-day: 1.38±0.54ng/ml) treatment. In the seven-day supplementation, no significant pre-post change was found with tea (change = -0.032±0.08ng/ml) compared to water (change = +0.003±0.10ng/ml) (Figure 5.10).

Figure 5.9 Effects of 7-day green tea supplementation on plasma HO-1 concentrations. Results are presented as mean±SD from 16 subjects

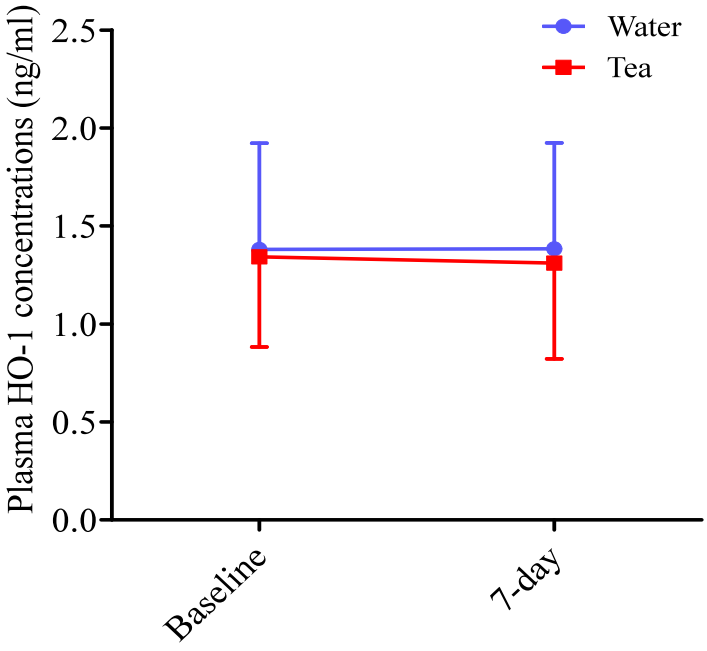
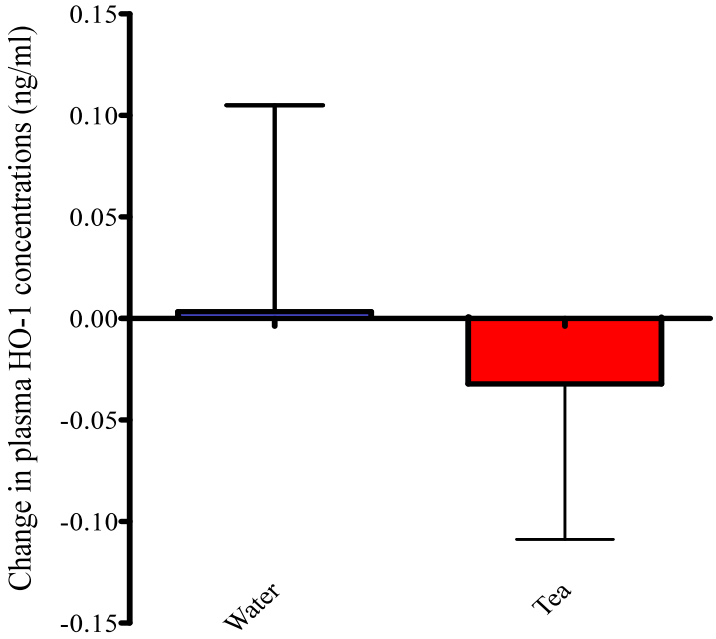


Figure 5.10 Change in plasma HO-1 concentrations between days 1 and 7. Results are presented as mean±SD from 16 subjects



Correlation of total antioxidant capacity, oxidation-induced lesions, hOGG1 activity and HO-1 protein expression with polyphenolic content in plasma

Plasma concentrations of catechins were measured by another member of our group (data shown in Table 5.6). Data on the plasma content of EGCG, EGC and ECG are shown, but the concentration of EC was below the detection limit of the method used. Both free and total catechins in plasma were correlated with total antioxidant capacity, oxidation-induced lesions, hOGG1 activity and HO-1 protein expression.

No significant correlations were found for total plasma antioxidant capacity and HO-1 protein expression with plasma catechin content. With regard to correlation with oxidation-induced lesions in DNA, significant ($p<0.01$) inverse associations were seen with both total (EGCG: $r= -0.4742$; ECG: $r = -0.4202$; EGC: $r= -0.4103$) and free catechins (EGCG: $r= -0.379$; ECG: $r = -0.3609$; EGC: $r= -0.4628$) (Figures 5.11, 5.12 & 5.13). Only total EGCG ($r= 0.6622$; $p<0.001$) and ECG ($r= 0.5857$, $p<0.01$) were found significantly correlated with lymphocytic hOGG1 activity (Figures 5.11 & 5.12). Correlations are presented in Table 5.7.

Table 5.6. Plasma concentrations of total and free catechins in acute and 7-day studies presented as mean(SD) and range in nmol/l

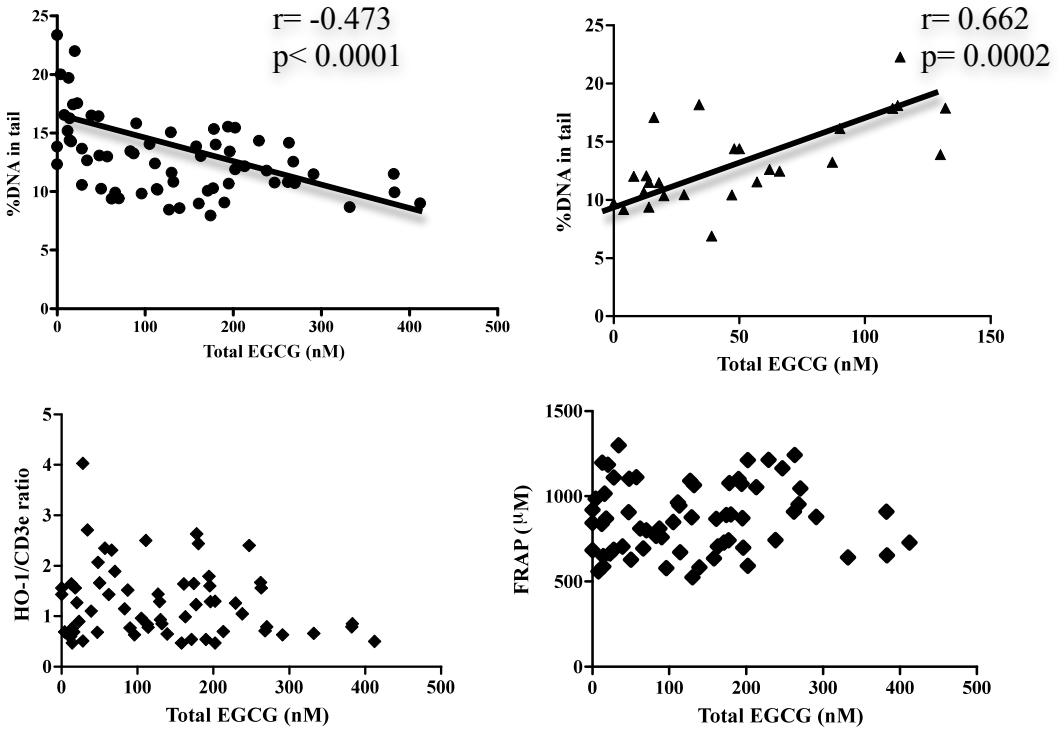
	Baseline		Post 60'		Post 120'		7-day	
	Tea	Water	Tea	Water	Tea	Water	Tea	Water
EGCG								
Free	3(8); 0-33	6(12); 0-48	310(117); 124-525	3(5); 0-14	213(117); 99-597	2(4); 0-14	10(8); 0-30	1(2); 0-7
Total	16(13); 0-47	20(15); 0-48	310(57); 105-332	17(14); 4-55	226(97); 96-412	15(16); 0-48	80(33); 28-132	15(16); 0-51
ECG								
Free	5(10); 0-40	7(8); 0-33	102(37); 61-196	5(6); 0-23	73(37); 41-182	3(3); 0-12	6(4); 0-12	3(3); 0-8
Total	41(28); 5-89	38(25); 4-93	134(51); 47-230	35(29); 0-112	138(61); 46-250	39(27); 4-92	116(55); 37-212	45(37); 3-127
EGC								
Free	Nil	Nil	56(28); 25-132	1(2); 0-8	32(13); 12-51	Nil	Nil	Nil
Total	Nil	1(3); 0-11	192(67); 99-327	Nil	169(98); 96-472	Nil	5(9); 0-29	1(5); 0-19

Table 5.7. Correlations between free and total catechins and biomarkers of interest

		Fpg lesions	hOGG1 activity	FRAP	HO-1 protein
EGCG	Free	r= -0.476 p<0.0001	n.s.	n.s.	n.s.
	Total	r= -0.473 p<0.0001	r= 0.662 p= 0.0002	n.s.	n.s.
ECG	Free	r= -0.385 p= 0.0017	n.s.	n.s.	n.s.
	Total	r= -0.385 p= 0.0017	r= 0.586 p= 0.0017	n.s.	n.s.
EGC	Free	r= -0.448 p= 0.0002	Nil	n.s.	n.s.
	Total	r= -0.440 p= 0.0003	Nil	n.s.	n.s.

Figure 5.11 Correlation between (a) Total and (b) Free EGCG with Fpg lesions (top left), hOGG1 activity (top right), HO-1 protein expression (bottom left) and FRAP (bottom right)

(a)



(b)

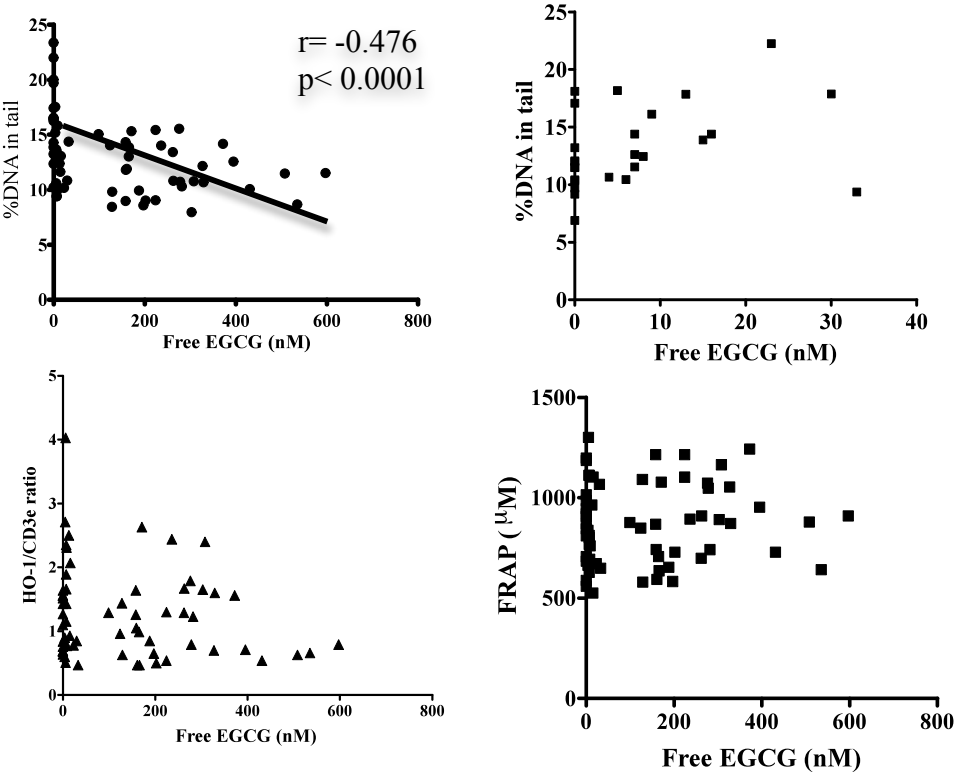


Figure 5.12 Correlation between (a) Total and (b) Free ECG with Fpg lesions (top left), hOGG1 activity (top right), HO-1 protein expression (bottom left) and FRAP (bottom right)

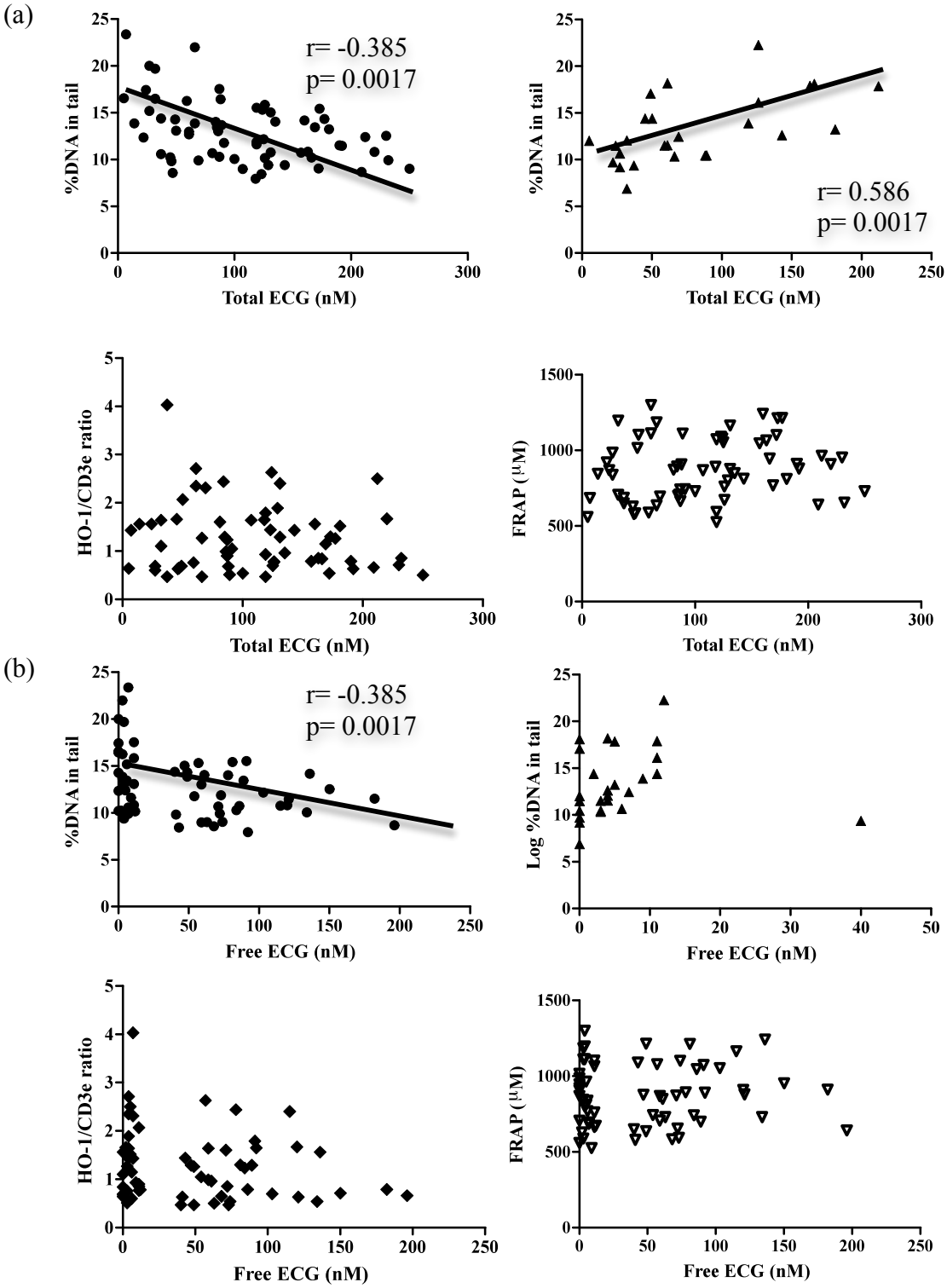


Figure 5.13 Correlation between (a) Total and (b) Free EGC with Fpg lesions (top left), hOGG1 activity (top right), HO-1 protein expression (bottom left) and FRAP (bottom right).

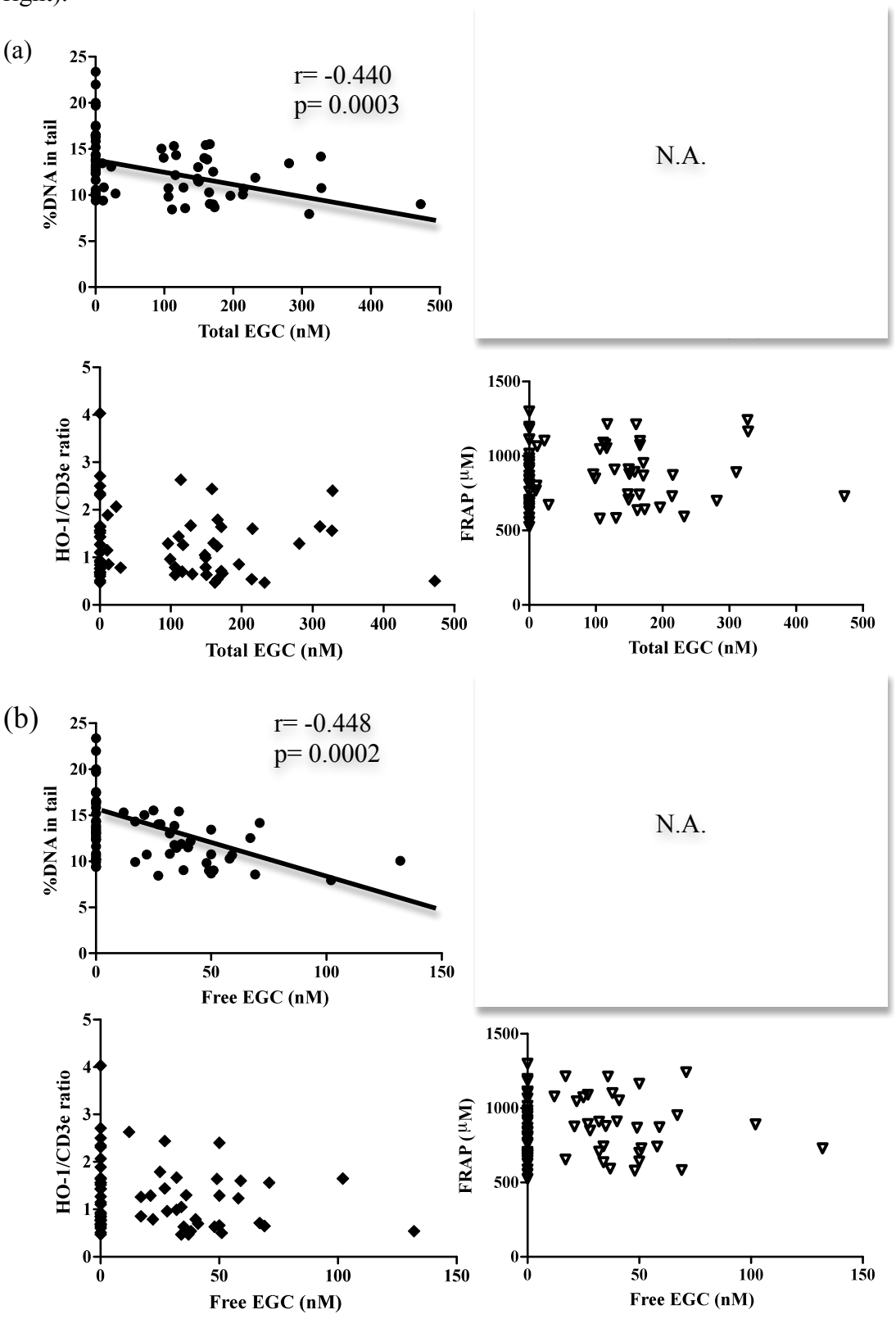


Table 5.8. Summary of main findings with ✓ showing significant differences between tea and water treatments

	Tests	Baseline	Post 60’	Post 120’	7-day
Heparinised Plasma (from heparinised blood)	FRAP	n.s.	n.s.	n.s.	n.s.
	Cholesterol	n.s.	n.s.	n.s.	n.s.
	HDL	n.s.	n.s.	n.s.	n.s.
	Triglycerides	n.s.	n.s.	n.s.	n.s.
	hsCRP	n.s.	nil	nil	n.s.
	Polyphenolic content	n.s.	n.s.	n.s.	n.s.
Lymphocytes (from heparinised blood)	Fpg lesions	n.s.	✓	✓	✓
	hOGG1 activity	n.s.	nil	nil	✓
	HO-1 Protein expression	n.s.	n.s.	n.s.	✓
RNA (from EDTA blood)	Gene expression for genes examined	n.s.	n.s.	n.s.	n.s.

Discussion:

Green tea has long been reported to have various health benefits, and these have been thought to be due to its high antioxidant content (Cabrera *et al*., 2006; Jain *et al.*, 2006; Williamson *et al.*, 2009). The underlying mechanisms of it health effects have been widely studied, but have proved to be elusive. However, with the demonstration of the pro-oxidant property of green tea, much attention has been paid to the possible redox mechanisms of green tea in addition to direct antioxidant protection (Halliwell & Gutteridge, 2007; Surh *et al.,* 2008; Benzie & Wachtel-Galor, 2010; Zhang *et al.,* 2010).

One suggested mechanism is adaptive cytoprotections induced via the redox-sensitive Nrf2/ARE signalling pathway, in which the hydrogen peroxide generated by green tea would trigger the translocation of transcription factor Nrf2 from cytosol to nucleus where it binds to the Antioxidant Response Element (ARE) to initiate the expression of a battery of cytoprotective products, i.e. Phase II detoxifying, antioxidant and DNA repair enzymes and HO-1 (Halliwell & Gutteridge, 2007; Surh *et al.,* 2008; Zhang *et al.,* 2010). *In vitro* and animal studies have been performed to demonstrate the ability of various phytochemicals with pro-oxidant property, such as catechins in green tea and resveratrol in red wine, to trigger the adaptive cellular response via Nrf2/ARE signalling pathway, and these phytochemicals are known as "ARE inducers" (Halliwell & Gutteridge, 2007; Surh *et al.,* 2008; Sriram *et al*., 2008; Benzie & Wachtel-Galor, 2010; Zhang *et al.,* 2010). To elucidate if this theory also applies in humans, this study aimed to examine if are any changes in gene and protein expression of ARE-driven products and changes in the activity of the DNA repair enzyme hOGG1 after a single dose (in the acute study) and

after a short period (7 days) of regular drinking of green tea. The effects of green tea consumption on total antioxidant capacity in plasma and amount of oxidation-induced lesions in lymphocytic DNA were also determined.

Results showed that single dose and regular consumption of green tea in the doses used did not lead to a significant increase in total antioxidant capacity in plasma as measured by a well-established method, the FRAP assay. There was a small, non significant increase in plasma antioxidant capacity in the acute study of ~2.5% at 60 minutes and 120 minutes after green tea consumption, and this increment was in agreement with results obtained from other human intervention studies (Hodgson *et al*., 2000; Leenen *et al*., 2000; Kimura *et al*., 2002; Rietveld & Wiseman, 2003). A 4% increase was found in an earlier study by our group, but it was noted that the dose was larger and stronger (400ml 5% w/v green tea) (Benzie *et al*., 1999b). The deviation was suggested to be influenced by various factors, including experimental design, dose, method used and ethnic and individual difference in absorption and metabolism of green tea (Sung *et al*., 2000; Kimura *et al*., 2002).

In this study, volunteers were required to maintain their own usual diets with addition of the supplement only, i.e. two extra cups of green tea or water per day. Therefore, it is also possible that their diet during the supplementation may have affected the results. Since this is a study aiming to evaluate the "actual" effect of green tea under usual diet and lifestyle, dietary restriction was not attempted, nor did we ask our volunteers to restrict their intake of anything in the lead up to the study. As previously reported by Kimura and

colleagues (2002), those interventions showing a significant increase in plasma antioxidant capacity were generally designed to take a higher consumption of polyphenolic content in green tea of 640-1500mg, which was suggested to be a "higher-than-normal" amount compared to around 100-300mg polyphenols estimated in a usual pattern of daily drinking of green tea.

Other studies have also demonstrated increase in catechin intake contributes to an increase in total antioxidant capacity in plasma (Sung *et al*., 2000). Consumption of green tea of the same concentration, an increase in total antioxidant capacity in plasma varied with different volumes of tea ingested (Sung *et al*., 2000). No significant increase was reported one and two hours after ingestion of 150ml green tea, but significant increase by around 6-12% was seen after ingestion of 300ml and 450ml green tea (Sung *et al*., 2000). Apart from the amount of catechin intake, metabolism of green tea catechins has also been thought to result in the deviation of plasma antioxidant capacity (Kimura *et al*., 2002; Henning *et al*., 2004). In particular, in a study investigating the effect of single/double and repeated ingestion of green tea extract on plasma catechin concentrations and antioxidant activity (n=16), it has been suggested that the bio-transformation of tea catechins, such as methylation, may lead to a decrease in antioxidant capacity by suppressing the ability to scavenge ROS and chelate metal ions, contributing to the deviations of the results of antioxidant capacity after drinking green tea in different studies (Kimura et la., 2002).

Even though the increase in plasma antioxidant capacity after drinking green tea was not statistically significant, levels of oxidation-induced lesions in lymphocytic DNA were found to be significantly decreased after a single dose and following 7 days' regular drinking of green tea. The Fpg-assisted comet assay is one of the widely used versions of the Comet assay to examine the levels of oxidation-induced Fpg lesions (Collins *et al*., 2004; Wong *et al*., 2005; Ho *et al*., 2011). As mentioned previously, Fpg causes single-stranded breaks for DNA repair and is specific to cleave oxidised purines, including 8-oxo-7,8-dihydroguanine (8-oxoGua), 2,6-diamino-4-hydroxy-5-formamido- pyrimidine (FaPyGua) and 4,6-diamino-5-formamidopyrimidine (FaPyAde) and other open-ring purines (Collins *et al*., 2004; Wong *et al*., 2005; Ho *et al*., 2011).

The observed decrease in the levels of oxidation-induced lesions implied that genoprotective effects could be achieved rapidly after green tea consumption. The DNA damage had decreased by approximately 31% at 60 minutes and 120 minutes after drinking the first cup of green tea, and this extent of decrease was maintained at the end of the 7 days' supplementation. The extent of decrease was in line with previous findings of our group, which examined the genoprotective effects of green tea after 4-weeks' supplementation (Han *et al*., 2011). The level of oxidation-induced lesions was found to be decreased by around 30% and 36% after 4-week supplementation of two types of green tea, Loong-cheng tea (the tea used here) and Screw-shaped tea, respectively (Han *et al*., 2011). This implies that the genoprotection of drinking green tea could be seen within an hour of consumption. The effect of a single cup of tea lasted for at least 2 hours and regular consumption, as shown here in this study for a week and our previous study

for 4 weeks, could help maintain the genoprotection of lymphocytic DNA against oxidation. Moreover, the consistency of the effect on oxidative lesions may suggest that the genoprotective effect of green tea may not be cumulative with regular consumption, and also could be related to reaching the maximal activity of DNA repair enzyme, one of the proposed mechanisms responsible for the genoprotection.

To achieve this genoprotection, several mechanisms have been proposed, including the direct antioxidant effect of green tea and the adaptive cytoprotective response of lymphocytes due to pro-oxidant properties of green tea (Halliwell & Gutteridge, 2007; Han *et al*., 2011). Although we have previously demonstrated (in Chapter 4) that hydrogen peroxide generated from green tea was not involved in the genoprotective effect of low dose green tea solution to pooled human lymphocytes incubated for 30 minutes *in vitro* and we have suggested the possibility of direct antioxidant effect on lymphocytes instead of adaptive cytoprotective response, it should be understood that direct comparison between the two studies is not appropriate. The metabolism of green tea in the human body is known to be much more complex than the *in vitro* environment and the generation of hydrogen peroxide by green tea *in vivo* has not yet been proven (Ogborne *et al.*, 2008; Del Rio *et al*., 2010; Sahin *et al*., 2010).

One of the speculated actions of green tea for its genoprotective effects on lymphocytes has been enhancement of the activity of the DNA repair enzyme, human 8-oxoguanine DNA glycosylase (hOGG1), involved in the initiation of base excision repair (BER), and human homologue of Fpg (Hakim *et al*., 2008). As with Fpg, hOGG1 recognises oxidised

lesions, both oxidised purines and apurinic/apyrimidinic (AP) sites, and converts these into single strand breaks (Collins *et al*., 2003; Collins & Azqueta, 2012). The activity of hOGG1 was measured by the cell extract version of the comet assay as described in Chapter 3. To recap, substrate (HeLa) cells with induced oxidised lesions are treated with lymphocytic extracts, and the hOGG1 in the extract then recognises the oxidised lesions and creates a certain amount of single strand breaks in a given time depending upon the hOGG1 activity in the extract. The higher the activity of hOGG1, the more single strand breaks would be formed in the lesions in the substrate cells, and so the higher would be the %DNA in tail as measured by the Comet assay.

Results showed a significant increase in DNA damage, i.e. %DNA in tail, in extract-treated substrate cells after 7-day's supplementation with green tea, and the difference was significant when compared to the control treatment. This reflects that an increased hOGG1 activity was achieved after drinking green tea for seven days. However, the change in gene expression of hOGG1 was not found to be significant after either single dose or regular drinking of green tea. It has been reported that hOGG1 promoter has similar properties to housekeeping genes, suggesting a consistent gene expression (Dhénaut *et al*., 2000; Radak & Boldogh, 2010). Besides, a poor correlation between hOGG1 mRNA expression and hOGG1 activity was demonstrated (Paz-Elizur *et al.*, 2007). Still, the activity of hOGG1 can be altered dynamically under various conditions (Radak & Boldogh, 2010). This could possibly be a result of post-translational modifications of the DNA repair enzyme, with modulation of the protein-protein interactions and the catalytic activity of the enzyme. hOGG1 is a DNA repair enzyme

involved in BER, and the most commonly reported post-translational modification on BER proteins is phosphorylation (Almeida & Sobol, 2007; Radak & Boldogh, 2010). Phosphorylation of hOGG1 was shown to be mediated by protein kinase C (PKC), cyclin-dependent kinase 4 (cdk4) and c-Abl tyrosine kinase (Dantzer *et al*., 2002; Hu *et al*., 2005; Radak & Boldogh, 2010). These mediators resulted in phosphorylation at different locations within hOGG1, regulating the activity of hOGG1. For example, phosphorylation at five different serine/threonine sites of hOGG1 enhanced incision activity on 8-oxoGua and AP lyase activity, whereas phosphorylation at tyrosine did not lead to changes in the activity (Hu *et al*., 2005; Alemida & Sobol, 2008; Radak & Boldogh, 2010).

In addition to phosphorylation, acetylation is another common modification demonstrated as a post-translational modification of hOGG1. This was shown to be involved mainly in the enhancement of the activity of hOGG1 (Bhakat *et al*., 2006; Radak & Boldogh, 2010). Acetylation of hOGG1 has been achieved by p300, a transcriptional co-activator, at acetyl acceptor lysine residues at positions 338 and 341, and has enhanced the activity of hOGG1 and also the displacement of acetylated hOGG1 by the downstream DNA repair enzyme, APE1 after excising AP sites demonstrated in various human cell lines, including colon carcinoma and primary mouse fibroblasts (Bhakat *et al*., 2006). The study also demonstrated the increased acetylation of hOGG1 upon the stimulation of exogenous oxidative challenge by treating cells with glucose oxidase for an hour without observing any changes in protein expression (Bhakat *et al*., 2006). However, it has also been reported that short exposure of human cells to a toxic heavy metal, cadmium, could

reversibly inhibit the hOGG1 activity via redox modifications of cysteine residues at positions 253 and 255 of hOGG1, suggesting another possible regulatory mechanism of the activity of hOGG1 (Bravard *et al*., 2006). These all are, at least in part, in agreement with our speculation that the beneficial effects of green tea are attributed to the mild generation of hydrogen peroxide in tea triggering particular adaptive mechanisms, i.e. the enhancement of hOGG1 activity possibly due to the post-translational modifications, in addition to direct antioxidant protection.

Results indicated that decreased DNA damage in lymphocytes after single dose and regular green tea supplementation was not only achieved by direct antioxidant protection, but also the involvement of increased hOGG1 activity after regular consumption driving more rapid repair of damaged DNA. Adaptive cellular response due to green tea consumption has also been thought to be involved in the genoprotective effects observed, and the redox-sensitive Nrf2/ARE signalling pathway was studied since green tea has been known to generate hydrogen peroxide *in vitro* and this property might be responsible as well for the observed increased hOGG1 activity. However, no detectable changes in the gene expression of the mediators and effectors of the signalling pathway, including *NRF2*, *KEAP1*, *BACH1*, *HMOX1*, *NQO1*, *GSTα*, and *XRCC5*, were found. Findings from various *in vitro* and animal studies investigating the effects of phytochemicals, including green tea catechins, on the Nrf2/ARE signalling pathway demonstrated that the protective effects of phytochemicals were, at least in part, attributed to the induction of the Nrf2/ARE signalling pathway, generally reflected by HO-1 expression and the nucleus translocation of transcription factor Nrf2 (Na *et al*.,

2008; Ogborne *et al*., 2008; Sriram *et al*., 2009; Akhlaghi & Bandy, 2010; Yang *et al*., 2012). EGCG, a major green tea catechin, was shown to be involved in the protection of primary vascular endothelial cells against polychlorinated biphenyl (PCB)-induced inflammation which contributes to cardiovascular diseases (Han *et al*., 2012). Pre-treatment of endothelial cells with EGCG for 3 hours was shown to counteract the increase of oxidative stress induced by PCB and this was associated with the activation of the Nrf2/ARE signalling pathway, in which gene expression of several Phase II antioxidant enzymes, such as GST and NQO1, was shown to be elevated dose-dependently to EGCG (Han *et al*., 2012).

Although the induction of Nrf2/ARE signalling pathway by phytochemicals has been widely reported in *in vitro* studies, findings have been conflicting in the limited human studies carried out (Wu *et al.*, 2006; Ogborne *et al.*, 2008; Benzie & Wachtel-Galor, 2010; Sahin *et al*., 2010; Zhang *et al.*, 2010). Gene expression of Phase II metabolising enzymes, e.g. NQO1 and HO-1, were significantly increased after three days of once daily oral sulphoraphane from 25-200g homogenised broccoli, suggesting the activation of Nrf2/ARE signalling pathway (Riedl *et al*., 2009). However, this was not in accordance with another human randomised cross-over study. The effects of steamed broccoli, rich in sulphoraphane, taken at 250g daily for 10 days on DNA damage and biomarkers of repair and defence enzymes were investigated on apparently healthy male smokers aged <30 years (n=27; Riso *et al*., 2010). The findings of this study showed that the decreased level of oxidation-induced lesions in lymphocytes was not associated with changes in the gene expression of repair and defence enzymes, including hOGG1 and

HO-1 (Riso *et al*., 2010). These, together with results showing a non-significant change in gene expression of the Nrf2/ARE signalling pathway in this current study suggest much more effort has to be paid in examining the possible role of the adaptive cytoprotection triggered by phytochemicals in humans, since sulphoraphane and polyphenolic catechins are well-reported "ARE inducers" in *in vitro* studies (Wu *et al.*, 2006; Ogborne *et al.*, 2008; Benzie & Wachtel-Galor, 2010; Sahin *et al*., 2010; Zhang *et al.*, 2010). Further studies unveiling the discrepancy among *in vitro*, animal and human studies are warranted.

Haem-oxygenase-1 (HO-1) is known to be a key effector of the Nrf2/ARE signalling pathway, and it is responsible for the degradation of haem (Wang & Chau, 2010; Abraham & Kappas, 2011; Kim *et al*., 2011). Haem degradation is considered a cytoprotective response since it involves the removal of pro-oxidant haem and the production of beneficial side products, which makes HO-1 a potential therapeutic funnel for various diseases, e.g. ischaemia and cardiovascular diseases (Wang & Chau, 2010; Abraham & Kappas, 2011; Kim *et al*., 2011). HO-1 is generally expressed at a low level in basal conditions, and is induced in response to cellular stress and oxidative stimuli (Wang & Chau, 2010). To elucidate if the adaptive cytoprotection could be triggered by acute and regular consumption of green tea, protein expression of HO-1 in lymphocyte extracts and plasma HO-1 concentrations were examined in addition to the gene expression of the Nrf2/ARE signalling pathway. Although no significant changes in gene expression were seen, the change in HO-1 protein expression in lymphocytes after regular green tea supplementation was significantly different from that after water control

treatment. Yet, no significant changes were observed in HO-1 protein expression after acute consumption of green tea or in plasma HO-1 concentrations in the single dose and 7 days studies. Results suggested that regular green tea consumption induces HO-1 protein expression, but that this induction did not result in an increase in plasma concentrations. This is possibly due to insufficient time for HO-1 induction after acute green tea consumption and regular consumption does not help increase the unknown pathway of entry (and role of) HO-1 into plasma.

Induction of HO-1 protein expression upon treatment with phytochemicals was reported in several *in vitro* and animal studies (Martin *et al*, 2004; Wu *et al*., 2006; Wung *et al*., 2006; Farombi *et al*., 2008; Pullikotil *et al*., 2012). Pullikotil and colleagues (2012) reported that EGCG could induce HO-1 expression at both gene and protein levels in human aortic endothelial cells after 8 hours at a concentration of 10μM and in mice treated five times a week for 5 weeks at a dosage of 75mg/kg body weight. The group indeed demonstrated the possible effect of EGCG to elevate HO-1 expression to inhibit the pro-inflammatory VCAM-1 expression which contributes to the tea-associated lowered risk of cardiovascular diseases (Pullikotil *et al*., 2012). Although HO-1 induction was shown in cells and animals, induction time and dosage required are still unclear. The deviations in the findings could possibly be due to the difference in experimental designs and the use and preparation of supplements. Particularly in human studies, more variations may be taken into account, and "GT" repeat polymorphism in HO-1 promoter in ARE and the individual's variability in terms of bioavailability of green tea catechins may possibly contribute to the deviation in findings (Henning *et al*., 2008; Del Rio *et al*.,

2010; Ferruzzi, 2010; Renouf *et al*., 2010; Wu *et al*., 2010; Katana *et al*., 2011; Chen *et al*., 2012; Choi *et al*., 2012).

It has been reported that the ability of individuals to elicit HO-1 induction and/or activity was varied due to the genetic differences at the HO-1 promoter region (Javanmard *et al*., 2011). This promoter region is highly polymorphic with the number of GT repeats, varying from 12 to 40 (Choi *et al*., 2012). In general, alleles with 23-25 GT repeats are defined as short (S) and those with more than 25 GT repeats as long (L). The number of GT repeats was suggested to determine the responsiveness of individuals by modulating the transcriptional activity of *HMOX-1*, where individuals with S/S genotype were suggested to be more resistant against oxidative stress and its related diseases, such as cardiovascular diseases and type II diabetes, due to its higher expression and activity of HO-1 (Hirai *et al*., 2003; Bao *et al*., 2010; Taha *et al.*, 2010; Wu *et al*., 2010; Katana *et al*., 2011; Chen *et al*., 2012; Choi *et al*., 2012). Chen and colleagues (2012) performed an association study of GT repeat polymorphism examining the risk of coronary heart disease (CHD) in Taiwanese. The group reported that the genotype of S/S in GT repeats was associated with a lower risk of CHD in subjects with higher level of oxidative stress, i.e. smokers, and the observation was further confirmed with a functional study using HUVECs endothelial cells with S/S genotype which showed that increase in HO-1 expression occurred only in response to oxidative stress (Chen *et al*., 2012). However, study by our group has not shown such an association (Choi *et al*., 2012). Both the high inter-individual variation in green tea metabolism and the "GT" repeat polymorphism in

HO-1 region may contribute to the deviations among studies focusing on the redox balance of phytochemicals via the Nrf2/ARE signalling pathway.

In addition to the individual's variation of "GT" repeats at the HO-1 promoter region, bioavailability of green tea catechins has also been widely studied but the results are conflicting and most studies reported a high inter-individual variability (Spencer, 2003; Williamson & Manach, 2005, Auger *et al*., 2008; Henning *et al*., 2008; Del Rio *et al*., 2010; Ferruzzi, 2010; Renouf *et al*., 2010). This may be attributed to poor gastrointestinal absorption, microbial gut population, rapid Phase II metabolism and urinary excretion, limiting the bioavailability and thereby affecting the biological effects of catechins (Williamson & Manach, 2005, Stalmach *et al*., 2009; Del Rio *et al*., 2010). Catechins would be either in free form or in conjugated form during metabolism. Among the four major green tea catechins, EGCG appears mainly in free form, but other catechins in plasma are found mainly in their sulphated and glucuronated forms, and exact physiological effects of these conjugated catechins are not well studied (Yang *et al*., 1998; Williamson & Manach, 2005; Del Rio *et al*., 2010; Renouf *et al*., 2011).

One of the objectives of this study was to investigate the possible correlation between plasma polyphenolic content and several biomarkers, including oxidation-induced DNA lesions, hOGG1 activity, total plasma antioxidant capacity, and HO-1 protein expression. Among the four catechins examined, only correlation with EC was not performed since it was not detectable in plasma.

Results showed that no significant correlations were found between catechins examined and total plasma antioxidant capacity, or with HO-1 protein expression. However, significant inverse correlations were observed between catechins and oxidation-induced Fpg lesions. Both total and free catechins measured were significantly correlated with the level of Fpg lesions. This implies free catechins alone and total catechins (in both free and conjugated forms) could contribute to a lower level of Fpg lesions, but further investigation will be needed to show if the same effect could be achieved by conjugated catechins alone. In addition, significant correlations were also found between total catechins (EGCG and ECG) and the activity of hOGG1, but not in the case of free catechins (EGCG and ECG). Since hOGG1 activity was examined only in samples from baseline and 7-day and the plasma total and free EGC levels at baseline and 7-day were mostly undetectable, correlation between hOGG1 activity and plasma EGC level was not performed. Increased total catechins were found to be correlated with increase hOGG1 activity, thereby enhancing DNA repair activity. The resulting correlations show that the hOGG1-enhancing activity could possibly be due to the conjugated catechins in plasma instead of free catechins. It is interesting to note that EGCG appeared mainly in free form in plasma, however, significant correlation was only observed in total EGCG content. Further study is warranted to investigate the possible actions of conjugated catechins, particularly EGCG, in plasma on the activity of hOGG1.

In summary, this is the first study examining the effect of green tea supplementation on redox balance via adaptive Nrf2/ARE signalling pathway and DNA damage and repair in human subjects, and was an extension of our *in vitro* work (as described in chapter 4).

Results indicate that consumption of green tea was associated with lowered DNA damage in lymphocytes, and that an acute effect was seen, and this was likely to be a direct antioxidant effect as no change in hOGG1 was seen and no evidence of ARE activation was observed. The same magnitude of genoprotection was seen in fasting samples after 7 days' regular intake. However, in the 7 day samples, at least some of the genoprotective effect is was likely to be due to increased DNA repair, as the activity of hOGG1 was increased, and although no changes in the gene expression of mediators involved in the Nrf2/ARE signalling pathways were seen, some evidence of ARE activation in the way of increased expression of HO-1 protein was observed in the 7 day samples.

Chapter 6
Summary of Main Findings, Limitations and Suggestions for Future Work, and Concluding Remarks

This last chapter is a recap and discussion of results in relation to the objectives stated in Chapter Two and the two parts of the study, and attempts to bring together the results and highlight novel findings from the *in vitro* and *in vivo* parts of the study, with brief discussion of the limitations and potential value and scope for development of the study findings.

Part 1 – *In vitro* study: acute (up to 120 minutes) effects of ingestion of a single dose of green tea in healthy human subjects

Objectives, findings and implications:

1. To measure the concentrations of hydrogen peroxide generated in tea solutions at 37^{o}C

   Hydrogen peroxide concentrations were measured in tea using the modified xylenol orange method. It was found that hydrogen peroxide was generated dose-dependently in the 0.005%, 0.01% and 0.05%w/v green tea solutions (in PBS), at 3.5μM, 7.3μM and 51.7μM, respectively, after 30 minutes incubation at 37^{o}C. The low levels of

hydrogen peroxide generated over 30 minutes would be unlikely to cause overt damage to cells, but that in the 0.05% tea is quite high. Based on our experience with the comet assay and cells subjected to a standard oxidant challenge with hydrogen peroxide, a level of ~50μM would be expected to create a large oxidant challenge and to generate significant levels of damage to DNA.

2. To investigate if green tea at low doses causes a change in:

a. Oxidation-induced DNA damage in human lymphocytes

Oxidation-induced lesions in lymphocytes were assessed using the Fpg-assisted comet assay. Without Fpg treatment, incubation of cells in the 0.05% tea solution caused a 4-fold increase in single strand breaks in lymphocytes compared to PBS treated cells, but no increase in damage was seen with tea at the lower doses tested (0.005% and 0.01%) . This is in line with the relative amounts of hydrogen peroxide generated in the different tea solutions. With Fpg treatment, a lower amount of DNA damage was found in lymphocytes incubated with 0.005% and 0.01% tea solutions compared to PBS-treated cells, indicating genoprotective effects of green tea *in vitro*. However, higher levels of damage were seen in lymphocytes incubated with 0.05% tea, compared to PBS control and the lower dose treated cells. This is in agreement with the high levels of hydrogen peroxide generated in 0.05% tea. Addition of CAT and both CAT and SOD to the tea solutions before the addition of cells prevented the increased DNA damage caused by 0.05% tea solution, but did not remove the genoprotective effect seen with the lower doses of tea. These new data suggest that the protection could be due to

direct antioxidant effects or to tea-induced cyto-adaptation(s), but that adaptions were not triggered by hydrogen peroxide.

b. Gene expression of key players in the Nrf2/ARE signalling pathway

Lymphocytes incubated in 0.005%, 0.01% and 0.05% tea solutions, with and without the addition of enzymes, showed no changes in *HMOX1*, *NRF2*, *KEAP1*, *BACH1*, and *hOGG1* genes as assessed by real time quantitative PCR. These results support the suggestion that protection by low dose tea was not mediated by a mild stress induced by small amounts of hydrogen peroxide.

If changes in *HMOX*-1 were seen, an additional objective was:

3. To correlate HMOX-1 expression with oxidation-induced DNA damage in human lymphocytes pre-incubated with green tea

However, no change in HMOX-1 gene expression was seen in the acute study.

Summary:

The overall aim of this part of the study was to examine the protective effects of green tea on human lymphocytic DNA and test the hypothesis that green tea can act as a pro-oxidant (by generating hydrogen peroxide) and cause changes in redox balance, leading to the activation of the redox sensitive Nrf2/ARE signalling pathway for downstream cytoprotection. This would also provide us with further insight into the role of hydrogen peroxide generated in green tea in the protective/damaging effects on lymphocytic DNA and the possible mechanism that hydrogen peroxide is involved in triggering the adaptive antioxidant response in cells to withstand a subsequent oxidant challenge. This part of the

study showed a dose-dependent generation of hydrogen peroxide from green tea. Green tea at low doses was shown to be responsible for the genoprotective effects on lymphocytic DNA, whereas hydrogen peroxide generated in green tea at high dose led to DNA damage which was mitigated with the addition of either CAT or CAT+SOD. The observed genoprotection possibly resulted from the direct antioxidant protection instead of adaptive cytoprotection since addition of CAT or CAT+SOD did not result in a change in genoprotection. Besides, the adaptive cytoprotective responses were not triggered at the gene expression level of several redox-sensitive genes.

2 – *In vivo* study: effect of 7 days' green tea supplementation in healthy subjects

Objectives, findings and implications:

To investigate changes in key variables *in vivo* in response to a single dose of green tea (acute response) in comparison to seven days' supplementation with green tea by examining if green tea supplementation:

1. Increases the gene and protein expression of key biochemical ARE products

No changes in gene expression of *HMOX*1, *NRF2*, *KEAP1*, *BACH1*, *NQO1*, *GSTα*, and *XRCC5* were observed after single dose and seven days' supplementation with green tea, using real time quantitative PCR. Although gene expression of *HMOX1* was not altered, HO-1 protein expression assessed by the Western blotting was increased after seven days' supplementation. Results (showing no change in gene expression) were similar to the finding in the acute study, showing no ARE activation and, by extension, no induction of oxidative stress-induced cyto-adaptations with

regular intake of green tea. However, the novel finding of an increase in the HO-1 protein level with regular intake of green tea is interesting. Its gene expression was not increased, but the amount of this cytoprotective protein was. It is possible that the 'window' of gene activation was missed (it may have been after the 2h period of study in the acute study) or that the half-life of HO-1 was increased, allowing some accumulation of the protein.

2. Increases plasma antioxidant status

Plasma total antioxidant capacity was examined using the Ferric Reducing/Antioxidant Power (FRAP) assay. Plasma antioxidant capacity increased slightly in the acute study, but did not significantly change after single dose or after seven days' supplementation with green tea.

3. Decreases lymphocytic DNA damage

Oxidation-induced lesions in lymphocytes were assessed using the Fpg-assisted comet assay. Both acute and regular intake of green tea resulted in a decrease in DNA damage level compared to baseline level. The decrease was ~30% in relative terms and was similar at 60 and 120 minutes after the single dose and in fasting plasma collected after 7 days of regular intake of green tea. These in vivo effects are similar to the in vitro genoprotective effects seen with low dose tea, and confirm our earlier findings in human subjects on green tea (Han et al., 2011). The effect could be due to direct antioxidant effects of absorbed catechins from tea, to endogenous protection of DNA against oxidant insults, or to improved DNA repair. While the clinical impact of

decreased DNA damage is not clear, it is reasonable to suggest that lower damage to DNA will have beneficial effects on health, and cellular health and functioning relies of DNA integrity.

4. Increases DNA repair of oxidative lesions

DNA repair enzyme hOGG1 activity was examined with the cell extract version of the comet assay. Since hOGG1 activity is not expected to change acutely, only samples collected after 7-day supplementation were assessed. A significant increase in hOGG1 activity was observed. These novel findings indicate that at least some of the genoprotective effects seen were due to improved DNA repair *in vivo* as a result of regular ingestion of green tea.

In addition, the effects were examined in relation to changes in plasma tea polyphenolic (catechins) levels at timed intervals after the single dose, and in fasting samples collected after 7 days of supplementation with green tea.

With regard to correlation with oxidation-induced lesions in DNA, inverse associations were seen with both total and free catechins (EGCG, ECG and EGC). However, only total EGCG and ECG were found to be positively correlated with lymphocytic hOGG1 activity. These results provide novel evidence indicating that EGCG and ECG, and in particular their conjugated forms, have genoprotective effects by enhancing DNA repair.

Summary:

This part of the controlled human supplementation trial was designed to investigate the

effect of regular green tea ingestion on redox balance via the adaptive Nrf2/ARE signalling pathway and DNA damage and repair. This study showed interesting and novel results, in that green tea consumption was associated with decreased lymphocytic DNA damage in both the acute and 7-day supplementation parts. Direct antioxidant effect and adaptive cellular responses have been thought to be responsible for the health benefits of green tea, and possibly other phytochemicals. One of the adaptive cellular responses investigated here was the change in the activity of the DNA repair enzyme hOGG1. This was shown to be increased in fasting samples after 7 days of supplementation with green tea, an effect that would likely make repair of oxidation-induced lesions more rapid or efficient, and thereby lowering baseline DNA damage, in line with the genoprotective effects seen in the day 8 Fpg comet assay results. The increase in hOGG1 activity may have been due to the redox-dependent post-translational modifications to thiol residues in the enzyme, or to redox-independent effects on, for example, phosphorylation status of the protein. Although these results were not associated with changes in gene expression of products involved in the adaptive Nrf2/ARE signalling pathway, HO-1 protein expression was found to be increased after regular green tea consumption. This may have been due to an extended half-life of the protein, and this could be responsible for some of the cytoprotective effects of regular consumption of green tea consumption.

Apart from the novel findings of the acute genoprotective effects of green tea and the enhancement of hOGG1 activity and HO-1 protein expression in lymphocytes with regular intake of green tea, this study is the first to show significant inverse correlations between catechin (EGCG, ECG and EGC) content in plasma and the level of oxidation-

induced Fpg lesions in lymphocytes, and a significant direct correlation between total (but interestingly not free) EGCG and ECG content in plasma and hOGG1 activity. We have shown that, following ingestion of green tea, free EGCG and ECG appear in plasma quite rapidly (Fung *et al.,* 2012), but that after regular ingestion the catechin profile changes. At 1-2 hours after ingestion of green tea, 100% of the EGCG and 50-75% of the ECG in plasma were in their free forms, whereas in fasting plasma after 7 days of supplementation, the figures fell to, respectively, <13% and <5% (Fung *et al.,* 2012), The total amount of EGCG and ECG in plasma in the day 8 fasting samples was low (averaging ~100nmol/l), but most was in the conjugated form (Fung *et al.,* 2012). Further study is needed on relative bioactivity of the conjugated and unconjugated catechins in relation to hOGG1 activity and DNA repair.

Limitations of the Study and Suggestions for Future Work

In the *in vitro* study, lymphocytes were harvested, pooled and treated with tea at 37^{o}C. Results from this *in vitro* study should not be directly interpreted as the "actual" physiological effects since oxidation conditions are different *in vivo* and *in vitro*, especially in relation to redox-sensitive genes. However, implications from the results could be applied to *in vivo* or other follow-up studies. Moreover, this study was based on a previous green tea study of our group and only one time point (30 minutes) was used to further investigate the possible role of hydrogen peroxide generated in green tea, and the incubation time may not be able to fully examine the effects of green tea on redox balance. Further study may be needed to investigate if redox-sensitive adaptations could

be triggered by prolonged tea incubation and it would be interesting to examine if protein expression of redox-sensitive agents, e.g. HO-1 is altered after a longer incubation time.

Sample size was one of the limitations in the supplementation study. Since there were no data available for the calculation of the required sample size for the examination of gene and protein expression, in this supplementation study, previous data on the Fpg-assisted version of the comet assay was used for the calculation of sample size. A larger study may be conducted to investigate the effect of green tea supplementation on the change in gene and protein expression of redox-sensitive ARE-driven products. In addition, only apparently healthy subjects were recruited, and effects may be different in those who are under increased oxidative stress at baseline due to age or disease, such as diabetes (Choi *et al.,* 2008). Moreover, it is possible that the genes of interest in this study may be tissue specific. Lymphocytes were the used sample in this study. These are the most easily accessible and most often used nucleated cells in human study, but the expression of the genes of interest may not be easily affected in lymphocytes, but may change in other tissues. Due to this possible tissue specificity of the genes of interest, it would be better if gene microarray were conducted to identify if there are some other genes which may have changed expression levels in lymphocytes after green tea supplementation. However, no resources are available for gene array testing in this current study. Further, it would be interesting to determine if post-translational modifications occurred in the proteins of interest (hOGG1 and HO-1). However, due to limited volume of blood and resource limitations, expression of phosphorylated proteins was not examined. Still, the novel findings revealed by this current study in terms of tea-induced increases in HO-1 protein

in lymphocytes without a concomitant increase in *HMOX-1* expression, along with tea-induced increases hOGG1 activity in lymphocytes without a concomitant increase in *hogg1* expression, provide the first evidence to support future study of tea supplementation-related post-translational effects on these important cytoprotective proteins.

Concluding remarks

There is an increasing burden of age-related diseases in Hong Kong and other developed societies (WHO, 2008). Increased oxidative stress has been associated with most age-related diseases (Anlasik *et al.*, 2005; Valko *et al.*, 2007). Increased oxidative stress is a result of ROS production, but, as well as being damaging agents, there is increasing evidence that some ROS (mainly superoxide and hydrogen peroxide) are crucial signalling molecules, and that cellular redox balance is one of the major determinants of cell signalling (Palmer & Paulson, 1997; Benzie & Wachtel-Galor, 2010). Green tea has been shown to decrease oxidative damage to DNA, but also to generate hydrogen peroxide *in vitro* (Halliwell & Gutteridge, 2007; Han *et al.*, 2011). The protective mechanism is not yet clear, though some might be due to direct antioxidant action by green tea polyphenols. However, decreased DNA damage seen previously with green tea could also be a result of enhanced DNA repair, or to cytoprotective cellular adaptations. The pro-oxidant effect of green tea may not be damaging but modulatory to cellular redox balance due to its mildness and consistency (when an antioxidant food is consumed regularly and when bioavailability is low). This in turn enables the antioxidant defence system to be more effective to a later, more severe oxidative challenge possibly by

enhancing DNA repair, and/or upregulating the expression or activity of endogenous antioxidants. This was the concept behind this study, in which *in vitro* and *in vivo* genoprotective effects of green tea were investigated, and in which acute and regular ingestion effects were examined. We explored the effects of green tea on expression of redox-sensitive genes and on some selected gene products, notably HO-1 and hOGG1. We also measured hOGG1 activity. The *in vitro* part showed that hydrogen peroxide generated in tea contributed to DNA damage at high dose, but did not appear to be responsible for the genoprotective effect of low dose tea, and no evidence of tea invoking the Nrf2/ARE signalling pathway was seen. The i*n vivo* (human supplementation) part showed that, in addition to the likely direct antioxidant protection, increased DNA repair was involved in the tea-associated genoprotection observed in lymphocytic DNA, even though no evidence of induction of the gene expression of the mediators of the Nrf2/ARE signalling pathway was found. Other possible mechanisms may thus be contributing to the enhancement of the activity of DNA repair enzyme hOGG1 and increased HO-1 that have less to do with changes in gene expression but relate to post-translation modifications to the functional proteins. Alternatively, green tea could bind or remove the effect of inhibitors of hOGG1 or enhance the provision of co-factors required for its activity and in some way extend the half-life of HO-1 within cells.

To conclude, this study has added to knowledge of how green tea can benefit health. Significant genoprotective effects were seen within 1 hour of tea ingestion, and the effect was maintained in fasting sample collected after 7 days of regular green tea intake. Though green tea was confirmed to generate hydrogen peroxide *in vitro*, this was not

responsible for its *in vitro* genoprotective effects, and no evidence of ARE-controlled gene activation was seen in either the *in vitro* or *in vivo* studies. The study showed that genoprotective effects correlated with EGCG and ECG levels in plasma, particularly conjugated EGCG and ECG. Interestingly the genoprotective effects of regular intake of green tea extended beyond simple antioxidant protection, as increased hOGG1 activity and HO-1 levels were seen. Therefore, this study provides novel science-based evidence for health promotion by green tea at the molecular and functional levels, and will support new avenues of research into diet and health promotion.

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Appendix 1:
Information sheet for volunteers

Acute and Short-Term Effects of Green Tea Ingestion on Redox Balance, DNA Damage and Repair in Controlled Human Study

……

You are invited to participate in a study conducted by Professor Iris Benzie and Mr. Cyrus Ho Kin Chun of the Department of Health Technology and Informatics at the Hong Kong Polytechnic University. Below is information on the study. A Chinese version of this is also available and Mr. Ho will be pleased to explain the details of study to you in English or Chinese.

1. **Purpose of the Study:**

   Ageing is associated with an increased risk of various diseases, and much attention is given to the use of natural products (such as herbs) and 'functional food', such as teas, many of which are high in antioxidants, for healthy ageing.

   The body is continuously exposed to potentially damaging agents (known as free radicals). The imbalance between the amount of free radicals and that of antioxidants in the body is known as oxidative stress. Oxidative stress may lead to diseases such as cancer, diabetes and heart disease. Antioxidant therapy, including increased consumption of antioxidant rich foods and beverages such as green tea, has been suggested as possibly beneficial in lowering the risk of developing these diseases.

   The purpose of this study is to examine the immediate effects (up to 2 to 3 h after drinking) of one cup of green tea, and the effects of drinking two cups of green tea every day for seven days on markers of oxidative stress. These markers will be measured in blood cells, plasma and urine collected before and after green tea ingestion so as to investigate if one dose or 7 days of regular intake of green tea.

   In this study we are particularly interested in protection of DNA against damage, the ability of cells to repair DNA damage, and the effects of green tea intake on certain genes that help control DNA repair and antioxidant defence in cells. We will also look at the amount of green tea antioxidants absorbed by measuring the levels of these products in blood and urine.

2. What the study involves and what you will be required to do:

This study is a controlled supplementation trial of cross-over design. This means that half the volunteers will supplement their normal diet with a fixed amount of green tea for seven days while other volunteers will be given hot water; blood and urine sample (collected before breakfast) will be collected before, during and after the supplementation period. This will be followed by 4 weeks of no supplement (usual diet only). Then the volunteers will 'cross-over' to take the other treatment for seven days, and blood and urine samples will be collected again at the beginning, during and at the end of the seven day period.

We will supply the tea and instructions.

Your part in the study will be over 6 weeks, and you will be required to come to PolyU on 4 pre-arranged 'test day' mornings. On two of the mornings you will be here for 3 hours (e.g. between around 8.30-11.30am). On the other two mornings you will be here around 20 minutes only.

Here is what will happen:
A blood and urine sample (fasting samples, collected BEFORE YOU HAVE BREAKFAST) will be collected between 8:30-9:30am, on a pre-arranged basis at our laboratory in PolyU. On two days, two additional blood samples (40 and 90 minutes after drinking tea) and two additional urine samples (at 90 and 180 mins after tea) will be collected.
After each blood taking session is completed, we will provide you with a light breakfast or lunch from the University canteen.

At the end of your part in the study we will provide you with HK$200 to cover your out of pocket expenses.

Please read the following information very carefully
As a volunteer for this study **you need to be able to say yes to the following points:**

1. You are aged between 35-50 years of age
2. You are in good general health and are not on any regular medications, have not been hospitalised in the past year, are not obese and do not have high blood pressure
3. You have not suffered a stroke or have other diseases such as heart disease, diabetes or cancer
4. You are not taking any antioxidant or herbal supplements on a regular basis and do not have special dietary preferences (for example, vegetarianism)

5. You do not smoke
6. You are not a regular (daily) green tea drinker
7. You agree to take 2 cups of green tea (made and taken as instructed; the green tea will be supplied) each day for seven days or 2 cups of water each day for seven days; then, after a four week period of your usual, non-supplemented diet, you will take 2 cups of water (if you took green tea before) or 2 cups of green tea (if you took water before) for seven days
8. On four occasions you will come to PolyU on a pre-arranged basis for blood and urine collection
9. You will have nothing to eat or drink (except water) for at least 10 hours before you come to PolyU on these days
10. You agree that on each of the four study days you will have blood collected from a vein in your arm and you will provide samples of urine
11. You agree that the first and third time you come to PolyU, we will collect a blood and urine sample from you before you drink a cup of green tea, and collect blood from you again at 90 and 120 minutes after you have drunk a cup of green tea. We will also collect a urine sample from you at 90 and 180 minutes. This part of the study is looking at the immediate ('acute') effect of drinking one cup of green tea
12. You agree that during the first and third days (during the 'acute' study), you will remain in PolyU until the last blood and urine sample has been collected (180 minutes after drinking the cup of green tea). You will not have anything to eat or drink except for small sips of water during this time
13. You agree that we will take 25mls of blood from a vein in your arm each time
14. You agree you will bring back any unused tea bags we have provided
15. When you return to PolyU on the second and fourth (last) occasion we will only collect one blood and urine sample from you before you have your breakfast
16. You understand your commitment to this project will last for around 6 weeks and requires you to come to PolyU between 8-9.30am on FOUR occasions for blood and urine collection
17. You agree that we can use your blood and urine samples in our laboratory tests of DNA damage, protection of DNA against damage, the ability of cells to repair DNA damage, and the effect of green tea intake on certain genes that help control DNA repair and antioxidant defences in the cell, and for signs of oxidative stress and to assess antioxidant defences
18. You realise that you can ask questions and can withdraw from the study at any time at no penalty.

19. You realise we will contact you by phone or email regularly to check you are taking the supplement (tea or water) and to remind you of your next blood collection appointment.
20. You agree to sign a consent form stating that you understand the information presented above and that you freely agree to take part in this study

IT IS VERY IMPORTANT THAT YOU FOLLOW YOUR USUAL DAILY DIETARY INTAKE DURING THE WHOLE STUDY.

THIS GREEN TEA SUPPLEMENT （OR WATER） ONLY ADDS TO YOUR USUAL DAILY DIETARY INTAKE, BUT NOT TO REPLACE ANYTHING IN YOUR DIET.

3. What is the risk of taking green tea?

There is no significant risk in taking green tea. The dosage we use is one of normal concentration.

4. What is the risk of blood sampling?

The testing should not result in any undue discomfort or harm to you. You may suffer a small bruise over the blood taking site; this is normal and will last only a day or two. Risk of infection is very small because we use new, sterile disposable syringes and needles, and the blood taking is performed by technicians experienced in this.

5. What will we do with your samples?

Your samples will be coded to protect your privacy, and only the research team will be able to break the code; keeping your information confidential is very important to us. After that, we will perform some tests on your samples to assess your antioxidant, oxidative stress balance, and the degree of DNA damage in your white blood cells before and after intake of green tea. This will give us information on how the human body responds to green tea supplementation in terms of indices of oxidative status.

If you volunteer, you have every right to withdraw from the study before
or during the measurement without penalty of any kind.

If you have any complaints about the conduct of this research study, please do not hesitate to contact Miss Kath Lui, Secretary of the Human Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University in person or in writing (c/o Research Office of the University).

If you would like more information about this study, please contact Mr Cyrus Ho, tel no 3400 8586 (hthkchun@) or **Prof. Iris Benzie** on tel. no. **3400-8572**.

Thank you for your interest in participating in this study.

Appendix 2:
Consent to participate in research

Acute and Short-Term Effects of Green Tea Ingestion on Redox Balance, DNA Damage and Repair in Controlled Human Study.

I _____ hereby consent to participate in the captioned research conducted by _____.

I understand that information obtained from this research may be used in future research and published. However, my right to privacy will be retained, i.e. my personal details will not be revealed.

The procedure as set out in the attached information sheet has been fully explained. I understand the benefit and risks involved. My participation in the project is voluntary.

I acknowledge that I have the right to question any part of the procedure and can withdraw at any time without penalty of any kind.

Name of participant:

_____

Signature of participant:

_____

Name of Parent or Guardian (if applicable):

_____

Signature of Parent or Guardian (if applicable):

_____

Name of researcher:

_____

Signature of researcher:

_____

Date:

_____

Appendix 3:
Instructions of tea preparation

Instructions:

Each subject will take the tea twice a day (preferably in the morning and evening) for seven days. Tea bags with weighed tea leaves and one measuring cups will be provided.

Preparations:

1. Boils water
2. Pours boiled water into the measuring cup provided (just reach 200ml)
3. Puts one tea bag into the hot water
4. Uses a plastic/glass spoon to stir the tea bag for around 3 minutes
5. Removes the tea bag from the tea using the spoon
6. Drinks the tea within 20 minutes

(Please return all the remaining tea bags after 1-week supplementation for statistical use, thank you)

Drinking Record:

	Day1	Day2	Day3	Day4	Day5	Day6	Day7
1st drink							
2nd drink							

Please add ✔ at suitable place after you have taken green tea/ hot water on that day.