The Hong Kong Polytechnic University

Department of Health Technology and Informatics

A STUDY OF GENOPROTECTIVE EFFECTS OF FUNCTIONAL FOODS/HERBS USING VARIOUS VERSIONS OF THE COMET ASSAY

By

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Certificate Of Originality

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Abstract of thesis entitled "A STUDY OF GENOPROTECTIVE EFFECTS OF FUNCTIONAL FOODS/HERBS USING VARIOUS VERSIONS OF THE COMET ASSAY" Submitted by WONG Wan Chi Vincy for the degree of Doctor of Philosophy at The Hong Kong Polytechnic University in June 2010

Abstract

Evidence shows that oxidative damage is a key factor in ageing and age-related disease and conceptually increased intake of dietary antioxidants may help to prevent oxidative damage *in vivo*, maintain DNA integrity, and so promote healthy ageing. DNA can also be damaged by oxidative and non-oxidative process involving ultraviolet (UV) irradiation, and this is a major cause of skin cancer in the elderly. The focus of herbs for health promotion is increasing worldwide, but scientific evidence of benefit is lacking, especially in regard to effects on DNA. The overall aim of this study was to assess the genoprotective and DNA repair effects of selected herbs, including *Ganoderma lucidum* (Lingzhi), *Camellia sinensis* (green tea), *Vaccinium myrtillus L* (bilberry) and *Cordyceps sinensis* (Cordyceps) by using various versions of the comet assay in human controlled trials and *in vitro* study. These herbs were selected because of their high reputation for health promotion and on published reports of potential benefit against oxidative stress or on DNA.

The main aim of the Lingzhi study was to investigate the acute post ingestion effect of Lingzhi on DNA repair by using the timed repair version of the comet assay. Plasma 'total' antioxidant capacity in plasma pre- and post-ingestion was also assessed by FRAP assay. These were assessed in a cross-over human intervention study, with blood samples collected from 7 healthy volunteers pre and 60 and 180 mins after single dose (3.3g) of Lingzhi. Results indicated that ingestion

of Lingzhi did not significantly affect the rate or efficiency of DNA repair of oxidation-induced damage. Lingzhi ingestion was shown to increase plasma antioxidant capacity slightly, however the increase was not statistically significant.

The main aims of green tea study was to investigate the genoprotective effect of regular intake of two green teas (Loongjin and Screw-shaped) on oxidative stress (using urine 8-oxodG as the biomarker, measured by LC-MS/MS) and DNA oxidation-induced damage (using the Fpg-assisted version of the comet assay). The relationship between these two biomarkers was also investigated. This trial was a single-blinded, multiple cross-over, placebo-controlled study, and blood and urine samples were collected from 18 healthy volunteers after 4 weeks' supplementation with each tea. Results showed that after supplementation of each tea, Fpg labile sites were ~30% less. However, no significant changes in urine 8-oxodG were seen and no significant correlation was seen between Fpg comet assay results and urine 8-oxodG results.

The specific aim of bilberry study was to investigate the effect of supplementation with bilberry (which is rich in anthocyanins) on base excision repair pathway in Type 2 diabetes patients using a lymphocyte extract version of the comet assay. This was assessed by a double-blinded, placebo-controlled cross-over intervention study, blood samples were collected from 20 Type 2 diabetes patients after 4 weeks' supplementation. Results showed no significant changes in hOGG1 activity after bilberry supplementation.

The specific aim of Cordyceps study was to investigate the genoprotective effects of polysaccharide-rich Cordyceps mycelial components against UVB-induced damage in normal human fibroblast cells. Cultured fibroblasts were pre-treated for 30 mins and 24 hours with defined concentration of hot water extracts of Cordyceps mycelia or mycelial exopolysaccharides. Fibroblasts were washed, irradiated with UVB, and immediately lysed, after which DNA damage was measured using the T4EV-assisted comet assay that detects predominantly a type of pyrimidine dimer (CPDs) that is a DNA lesion produced specifically by UVB. Fibroblasts showed significant ($P<0.01$) downward trend in DNA damage with increasing concentrations of each extract for both 30 mins and 24 hrs pre-incubation treatments.

Numerous mechanisms are involved in genoprotection, including cytoprotective adaptation, antioxidant property and upregulating DNA repair capacity. Lack of genoprotective effect in particular mechanism does not mean it has no beneficial effect (Lingzhi and bilberry studies). Precaution also should be taken to avoid overstating the genoprotective effect of green tea and Cordyceps, since the extact mechanisms behind the effects are still unclear. Nonetheless, the new data presented here are supportive of green tea against oxidation-induced DNA damage, and are also supportive of Cordyceps polysaccharides against non-oxidation-induced DNA damage caused by UVB.

List Of Published And Presented Work From This Thesis

Journal Articles

Han KC, **Wong WC**, Benzie IFF (2010). Genoprotective effects of green tea (Camellia sinensis) in human subjects: results of a controlled supplementation trial. *Br J Nutr*. (Sept E-pub ahead of print) Doi: 10.1017/S0007114510003211.

Wong WC, Wu JY, Benzie IFF (2010). Photoprotective potential of Cordyceps polysaccharides against ultraviolet B radiation-induced DNA damage to human skin cells. Revised version submitted to *J Am Acad Dermatol.*

Wachtel-Galor S, **Wong V**, Choi SW, Benzie IFF (2010) Antioxidant power and DNA repair effects of Lingzhi or Reishi medicinal mushroom, *Ganoderma lucidum* (W.Curt.:Fr.)P. Karst. (Aphyllophoromycetideae) in human acute post-ingestion study. Accepted by *Int J Medicinal Mushrooms*.

Conference Papers

Wong VWC, Wachtel-Galor S, Benzie IFF. Acute post-ingestion effect of *Ganoderma lucidum* on DNA repair. World Congress on Chinese Medicine, Nov 23-25 2006, Hong Kong, pp173.

Wong WC, Wachtel-Galor S, Benzie IFF. Acute effect of *Ganoderma lucidum* (Lingzhi) on human lymphocytic DNA repair results of a controlled cross-over trial. Scientific Meeting of the Nutrition Society, Coleraine, July 2007, UK.

Benzie IFF, Han KC, **Wong WC**. Effects of green tea on lymphocytic DNA damage and urinary 8-hydroxy-2'-dexoyguanosine excretion in a controlled human intervention trial. 'Food and Function' International Scientific Conference on Nutraceuticals and Functional Foods, June 9-11 2009, Žilina, Slovakia, pp13 (oral presentation).

Han KC, **Wong VWC,** Benzie IFF. Regular intake of green tea protects DNA from oxidative damage, but not through effects on the nucleotide pool: results of a controlled human intervention. 5th Joint Meeting of The Societies for Free Radical Research Australasia and Japan held in conjunction with the Mutagenesis and Experimental Pathology Society of Australia, Dec 1-4 2009, University of Sydney, Australia (oral presentation).

Wong VWC, Wu JY, Benzie IFF. A study of protective effects of Cordyceps against ultraviolet radiation-induced DNA damage to human skin cells. 5th Joint Meeting of The Societies for Free Radical Research Australasia and Japan held in conjunction with the Mutagenesis and Experimental Pathology Society of Australia, Dec 1-4 2009, University of Sydney, Australia, pp 128.

Wong WC, Wu JY, Benzie IFF. Polysaccharides from *Cordyceps sinensis* mycelial cultures can protect human skin cells against ultraviolet B radiation. Abstract presented in The 4th International Functional Foods Symposium Oct 29-30 2009, Hong Kong.

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List Of Abbreviations

<500EPSCM	Exopolysaccharides of molecular weight <500kDa
•OH	Hydroxyl radical
$^1\Sigma_gO_2$	Singlet oxygen
6-4 PPs	(6-4) pyrimidine-pyrimidinone photoproducts
8-OHdG	8-hydroxy-2'-deoxyguanosine
8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine
8-oxoGua	8-oxo-7,8-dihydroguanine
A	Adenine
AlkA	Methyladenine DNA glycosylase II
ALL	Lymphoid leukemia
ALS	Alkaline labile sites
AML	Acute myeloid leukemia
AP site	Apurinic or apyrimidinic sites
ARE	Antioxidant Response Element
ATL	T cell leukaemia lymphoma
ATP	Adenosine triphosphate
BER	Base excision repair
BMI	Body mass index
BSA	Bovine serum albumin
C	Cytosine
CBMN	Cytokinesis block micronucleus assay
CID	Collision induced dissociation
CPDs	Cyclobutane pyrimidine dimers
CYT-B	Cytochalasin –B
DAPI	4,6-diamidino- 2-phenylindole
dG	Deoxyguanosine
dGDP	Deoxyguanosine diphosphate
dGTP	Deoxyguanosine triphosphate
DM	Diabetes mellitus

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double strand breaks
EC	Epicatechin
ECD	Electrochemical detector
ECG	Epicatechin -3-gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbant assay
EMEM	Eagle's Minimum Essential Medium
Endo III	Endonuclease III
ETC	Electron transport chain
FapyAde	4,6-diamino-5-formamidopyrimidine
FapyGua	2,6-diamino-4-hydroxy-5-formamidopyrimidine
FBS	Foetal bovine serum
Fe^{3+}/TPTZ	Ferric-tripyridyltriazine
Fpg	Formamidopyrimidine glycosylase
FRAP	Ferric Reducing Antioxidant Power
G	Guanine
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Gluatathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HbA_{1C}	Glycosylated haemoglobin
HCl	Hydrochloric acid
HGSIL	High grade squamous intraepithelial lesion
hMTH1	8-oxo-dGTpase
HOCl	Hypochlorous acid
hOGG1	8-oxoGua DNA glycosylase 1

HPLC	High performance liquid chromatography
HWECMyc	Hot water extract of Cordyceps mycelia
IFN	Interferon
IL	Interleukin
KCl	Potassium chloride
KOH	Potassium hydroxide
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low-density lipoprotein
LGSIL	Low grade squamous intraepithelial lesion
LMP	Type VII low gelling point agarose
m/z	Mass to charge ratio
MDS	Myelodysplastic syndrome
MMR	Mismatch repair
Na_2EDTA	Disodium ethylenediaminetetracetic acid dihydrate
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NER	Nucleotide excision repair
NF-κB	Nuclear factor-kappa B
NIR	Nucleotide incision repair
NO_2•	Nitrogen dioxide
O_2•$^-$	Superoxide radical
O_3	Ozone
PARP-1	Poly (ADP-ribose) polymerase-1
PBS	Phosphate buffered saline
PDA	Photodiode array
ROO•	Peroxyl radical
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SPE	Solid phase extraction
SSB	Single-strand breaks

T	Thymine
T4EV	T4 endonuclease V
TNF	Tumour necrosis factor
TPA	Tetradecanoylphorbol-13-acetate
Tris	Tris(hydroxymethyl) aminomethane
UDG	Uracil DNA glycosylase
UV	Ultraviolet
XP	Xeroderma pigmentosum
XPC	Xeroderma pigmentosum complementation group C
XPF	Xeroderma pigmentosum complementation group F

Chapter 1

LITERATURE REVIEW

Introduction

The population in developed countries is ageing, and this brings a severe burden of chronic, debilitating disease to our society. Around 30 % of the global population will be aged >65 years by the year 2050 (Joseph *et al.*, 2009). Aged people commonly suffer from one or more chronic diseases, such as cancer (Halliwell, 2007), Alzheimer's Disease (Joseph *et al.*, 2009), cardiovascular disease (Finkel & Holbrook, 2000) and diabetes mellitus (DM) (Chow *et al.,* 2005). Treatment for age-related disease is expensive, harsh in the case of cancer and generally unsuccessful. Therefore, population ageing results in increasing demand for and burden of healthcare (Yip & Law, 2002; Yach *et al.*, 2004). These are key issues, and consequently, there is great interest and potential value in the investigation of strategies that are designed to promote health (rather than treat disease). In Asia, and increasingly in western countries, there is a large focus on traditional herbal medicines, and there are also many functional foods that are valued for health maintenance and the promotion of functional longevity. These include *Ganoderma lucidum* ('Lingzhi') (Wachtel-Galor *et al.*, 2004c), *Camellia sinensis* ('Green tea') (Benzie *et al.*, 1999), *Cordyceps sinensis* (Paterson, 2008) and *Vaccinium Myrtillus L* (Bilberry) (Zafra-Stone *et al.*, 2007), and their usage is common. However, as for most herbs, objective scientific evidence of health benefit is lacking (Opara, 2004). This is also a key issue.

In age related diseases, and perhaps the ageing process itself, a key factor is believed to be oxidative damage to biomolecules ('oxidative stress') such as deoxyribonucleic acid (DNA), protein and lipid (Benzie, 2000; Halliwell, 2002; Bartsch & Nair, 2004). Oxidative stress is caused by reactive oxygen species (ROS) which are formed inevitably and continuously within the human body (Halliwell, 2009). Increased oxidative stress is caused by inflammation (Halliwell, 2009), diabetes mellitus (Rahimi *et al.*, 2005), and by some drugs and environmental agents, such as ultraviolet radiation (Cleaver & Crowley, 2002) and cigarette smoke (Marwick *et al.*, 2002). UV radiation can also cause DNA damage by non-oxidative processes (Kielbassa *et al.*, 1997; Cleaver & Crowley, 2002; Bens, 2008; Hakozaki *et al.*, 2008). Conceptually, if oxidative and other damage to DNA and other key biomolecules can be attenuated then risk of age-related disease may be lowered and health promoted. Similarly, if DNA repair can be enhanced then cellular metabolism and tissue function may be maintained in a healthy state. If lifestyle factors, such as exercise, or dietary agents, such as herbs, could be shown to demonstrate antioxidant, DNA repair enhancing or genoprotective effects in objective study, this would provide scientific evidence to support their use in health promotion and lead to better understanding of their mechanisms of action. This is the focus of this study, which addresses the problem of designing and evaluating effective strategies for health promotion in our ageing population, with a focus on DNA damage and repair using different models of the comet assay.

DNA Integrity And Health

DNA is the biomolecule of genes (Halliwell, 2000). DNA is located in the nucleus and mitochondria and is composed of two strands of nucleosides consisting of deoxyriboses and nucleotide bases linked by phosphodiester bonds. The two strands of DNA are anti-parallel and contain four different nucleotide bases, which are adenine, guanine, thymine and cytosine (Watson & Crick, 1953). DNA bases are paired across the two strands by hydrogen bonds in a special pattern: adenine (A) is paired with thymine (T) and guanine (G) is paired with cytosine (C) (Hannon, 2007). DNA integrity and the maintenance of genomic stability are crucial factors for health. However, ROS, ultraviolet light and other agents can damage both nuclear and mitochondrial DNA (Rass & Reichrath, 2008; Halliwell, 2009). This can lead to cell death (by apoptosis), less adenosine triphosphate (ATP) production, altered phenotypic expression and carcinogenic mutations (Hollander & Fornace, 2002; Gruber *et al.*, 2008).

If dramatic, DNA damage kills the cell and thus can lead to irreplaceable loss of non-mitotic tissue. Smaller amounts of DNA damage may be tolerated by the cell, but lesions should trigger DNA repair (Sancar *et al.*, 2004). Usually, the cell cycle is halted by p53 protein at the G1 phase until the repair is completed. However, some DNA lesions may be replicated without being recognized by the repair system (Madhusudan & Middleton, 2005). In such cases, small changes and mutations can accumulate in daughter cells and lead to a decline in function due to altered homeostasis. If mutations occur in genes related to the rate of cell proliferation, such

as tumour suppressor genes or proto-oncogenes, the risk for cancer development may be increased. Therefore, DNA damage and repair is an area of increasing interest (Halliwell, 2002; Collins *et al.*, 2004; Halliwell, 2007; Møller *et al.,* 2008).

The Causes And Types Of DNA Damage

The mechanisms of DNA damage have been extensively studied. DNA can be damaged in several different ways, and by different agents or processes. There can be formation of apurinic or apyrimidinic (AP) sites, and oxidation of purines and pyrimidines which will be referred to here as simple base damage. DNA damage can also be in the form of single-strand breaks (SSB), double strand breaks (DSB), DNA adduct formation, formation of pyrimidine dimers and insertion or deletion loops (Evans *et al.*, 2004). These various types of damage can be caused directly by some ROS, but also indirectly or directly by other factors (Cleaver & Crowley, 2002). For example, DNA changes or damage can be induced by non-oxidative processes, such as insertion of viral genes, formation of dimers by ultraviolet B light, adduct formation by carcinogens, and lack of DNA repair will allow changes to accumulate (Møller *et al.,* 2008; Rass & Reichrath, 2008; Patient *et al.*, 2009).

In relation to oxidation-induced damage, different ROS react with DNA in different ways (Halliwell & Gutteridge, 2007). For example, the hydroxyl radical (•OH) readily reacts with all four DNA bases (though most easily with guanine); by comparison, hydrogen peroxide (H_2O_2) does not attack DNA under normal

physiological conditions (Halliwell & Aruoma, 1991; Evans *et al.*, 2004). Singlet oxygen ($^1\Sigma_g O_2$), a non-radical form of 'energized' oxygen can react with DNA, but the reaction is limited to guanine bases (Halliwell, 1999).

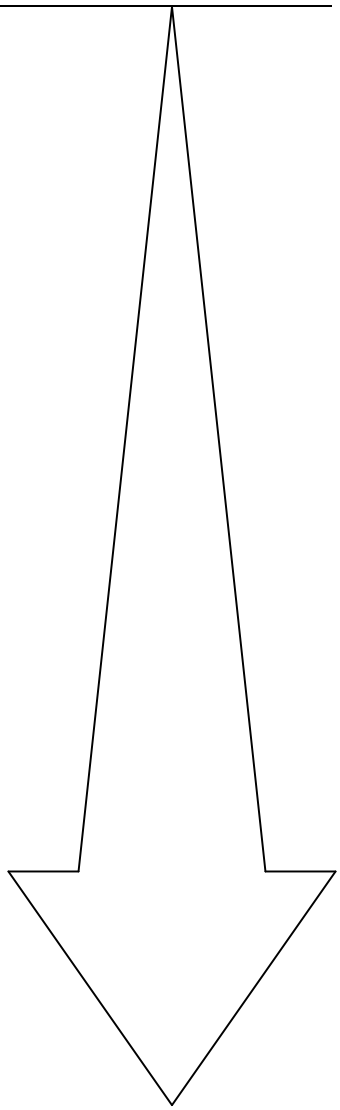
At physiological pH DNA is anionic because it is rich in phosphate groups, and it readily binds cations such as Fe^{3+} and Cu^{2+} (Halliwell & Gutteridge, 2007). This may increase the chance for oxidative attack by hydroxyl radicals generated by the Haber-Weiss reaction, especially at sites of guanine bases, since Cu^{2+} binds to guanine more readily than other bases (Halliwell & Gutteridge, 2007). In addition, due to its lower redox potential (E^o = 1.29 V compared to 1.42 V for adenine (Steenken & Jovanovic, 1997), guanine is the most easily oxidized base (Niles *et al.*, 2006), and so most of the oxidized bases formed within the body will be oxidized guanines. It is estimated that there are at least a few hundred oxidative hits in each human body cell per day (Halliwell, 2000).

The various types of ROS and their sites of production are outlined in Table 1.1. Only the most reactive of these (hydroxyl radical and peroxynitrite) are able to directly oxidize DNA bases (Steenken & Jovanovic, 1997). The way in which DNA is damaged by hydroxyl radical and ultraviolet (UV) irradiation is described in more detail below, as oxidation-induced and UV induced damage were of interest in this study.

Table 1.1. Various types of ROS and their sites of production.

(Adapted and modified from Table 1 in Strain JJ, Benzie IF. Diet and Antioxidant Defence. In: Sadler M, Strain JJ, Cabellero B, eds. The Encyclopedia of Human Nutrition. London: Academic Press, Oxford. 1999: 95-106.)

Name	Formula or Symbol	Origin	Radical or Non-Radical	Relative Reactivity
Molecular oxygen	O_2	Atmosphere	Radical	
Nitric oxide	NO•	Endothelial cells	Radical	
Nitrogen dioxide	NO_2•	Cigarette smoke; Industrial pollutants	Radical	
Superoxide	O_2•$^-$	Electron transport chain; Oxidative burst of phagocytes	Radical	
Peroxyl	ROO•	Reaction of carbon-centred radical with O_2 Break down of organic peroxides	Radical	
Singlet oxygen	$^1\Delta_gO_2$ $^1\Sigma_gO_2$	Photosensitization reactions of riboflavin, bilirubin and retinal	Non-radical	
Hydrogen peroxide	H_2O_2	Oxidative burst of phagocytes From dismutation of O_2•$^-$ by superoxide dismutases (SOD)	Non-radical	
Alkoxyl	RO•	Reaction of carbon-centred radical with O_2 Break down of organic peroxides	Radical	
Hypochlorous acid	HOCl	Oxidative burst of phagocytes	Non-radical	
Peroxynitrite	HNOO•	Reaction of NO• with O_2•$^-$	Non-radical	
Hydroxyl	•OH	Fenton/ Haber-Weiss reaction Radiation-induced fission of peroxides (including H_2O_2) and water	Radical	



Reaction Of Hydroxyl Radical (•OH) And Hydrogen Peroxide (H_2O_2) With DNA

The •OH radical is highly reactive. The •OH radical can generate various products with DNA by abstraction of electrons or addition reaction (Evans *et al.*, 2004). Due to the high reactivity, •OH cannot diffuse far and only induces DNA damage when generated near to or on it. There have been two mechanisms proposed to explain why •OH is generated near DNA. First, copper ions are usually present in GC rich sequences, increasing the chance of Haber-Weiss reactions (Halliwell & Gutteridge, 2007). Second, oxidative stress results in an increase of free intracellular iron or copper ions, which enhance their binding to DNA (Halliwell & Aruoma, 1991). In addition, H_2O_2 can pass through the cell membrane and freely diffuse through the cell, and generates •OH by the Haber-Weiss or Fenton reaction (Wang, 2008).

The •OH radical can react with guanine and adenine (purines) at C-4, C-5 and C-8 in the purine ring. Due to its high mutagenic activities, the C8 oxidation product of guanine, 8-oxo-7,8-dihydroguanine (also referred to as 8-oxoguanine, or 8-oxoGua), and its deoxynucleoside, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), are most well studied. The guanine C8-OH-adduct radical is the first product after •OH reacts with C-8 of guanine (Figure 1.1). The guanine C8-OH-adduct radical can be reduced to 7-hydro-8-hydroxy-2'-deoxyguanosine, or oxidized with formation of 8-oxodG, which can rearrange to 8-OHdG (also referred to as 8-hydroxy-2'-deoxyguanosine) (Evans *et al.*, 2004; Valavanidis *et al.*, 2009). •OH reacts with thymine and cytosine (pyrimidines) by adding to the C-5 and C-6 double

bond to form 5-hydroxy-6-yl or 6-hydroxyl-5-yl radicals (Evans *et al.*, 2004). Besides, •OH also reacts with nuclear protein to form protein radicals. DNA-protein cross-links form when a protein radical meets with a DNA base radical (Evans *et al.*, 2004).

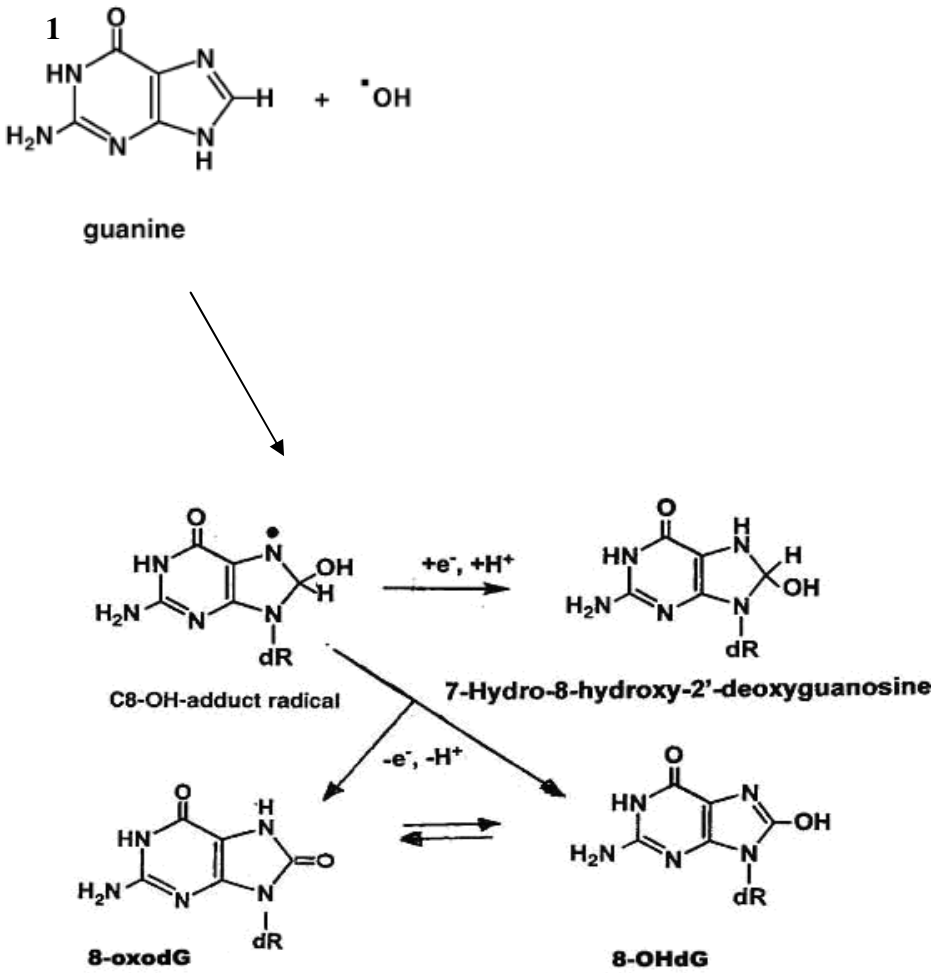


Figure 1.1. Oxidation of guanine and its products; formation of 8-oxodG from C8-OH-adduct radical.
(Adapted and modified from Figure 1 in Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2009;27(2):120-39.)

Ultraviolet (UV) Irradiation And DNA

UV irradiation is composed of UVA (320-400nm), UVB (290-320nm) and UVC (200-290nm) (Cadet *et al.*, 1997; Sinha & Häder ,2002; Hiraku *et al.*, 2007). The type of DNA damage induced by UV is wavelength dependent (Figure 1.2). UVA induces DNA damage indirectly by type I and type II photosensitizations. In type I reaction, after a photosensitizer absorbs photons, the excited photosensitizer can transfer the energy to DNA via electron transfer (Kielbassa *et al.*, 1997). Type II reaction involves generating single oxygen or superoxide by energy transfer from an excited photosensitizer to oxygen molecules (Hiraku *et al.*, 2007; Rünger & Kappes, 2008). Superoxide can generate •OH via the Fenton reaction. UVA also can induce some direct, non-oxidative damage of the type induced by UVB (as described later).

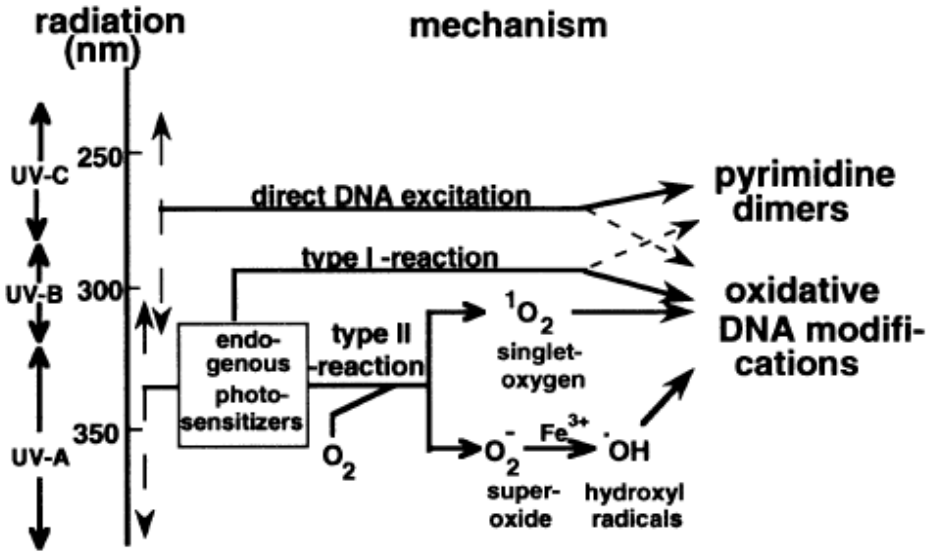


Figure 1.2. Mechanisms of UV-induced DNA damage at different wavelengths. *(Adapted from Figure 1 in Kielbassa C, Roza L & Epe B. Wavelength dependence of oxidative DNA damage induced by UV and visible light. Carcinogenesis 1997;18: 811-816.)*

The maximum UV absorption of DNA is at 260 nm, and absorption diminishes with increasing wavelength (Rünger & Kappes, 2008). UVB and UVC can be directly absorbed by DNA, creating characteristic types of DNA damage, the cyclobutane pyrimidine dimers (CPDs) and the (6-4) pyrimidine-pyrimidinone photoproducts (6-4 PPs) (Figure 1.3). Both types of dimer are generated at di-pyrimidine sites within the DNA strand and cause C → T transition mutations (Ichihashi *et al.*, 2003; Marrot & Meunier, 2008) . When compared with CPDs, 6-4 PPs seem less mutagenic, since most of 6-4 PPs are repaired within 3 hours after UVB irradiation, while CPDs need more than 9 hours to repair (Nakagawa *et al.*, 1998). The DNA damage induced by UV light is strongly associated with photoageing and skin cancer (Rass & Reichrath, 2008).

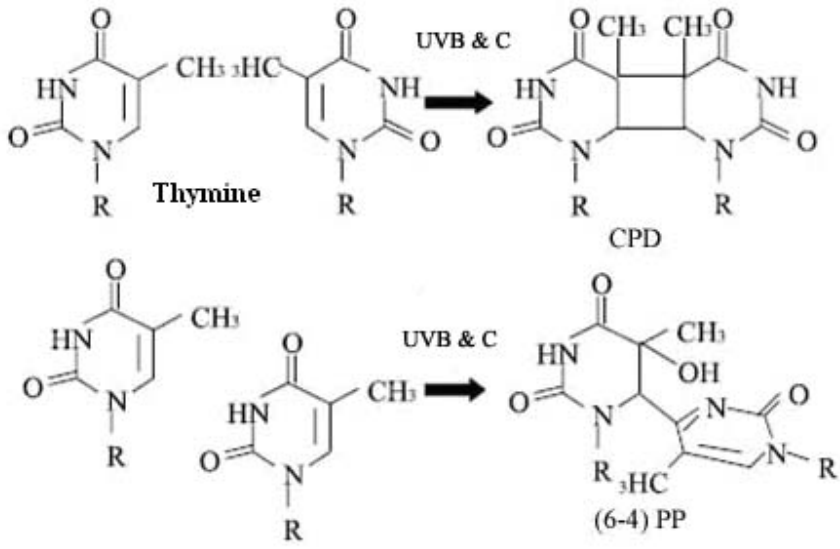


Figure 1.3. Formation of CPD and 6-4 PP by joining of adjacent di-pyrimidine bases within the same strand.
(Adapted and modified from Figure 1 in Ichihashi M, Ueda M, Budiyanto A, Bito T, Oka M, Fukunaga M, Tsuru K & Horikawa T. UV-induced skin damage. Toxicology 2009; 189, 21-39.)

Repair Of DNA Damage

Fidelity in DNA and accurate transmission of genetic material to daughter cells is essential to maintain normal gene expression and cell growth and metabolism. The human genome is continuously monitored by DNA repair systems. Once the DNA lesion is detected, the repair system restores the DNA via different repair pathways, depending on the type of damage. Pathways include base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) (Christmann *et al.*, 2003; Madhusudan & Middleton, 2005). BER and NER are responsible to repair damaged DNA before DNA replication, and MMR pathway is responsible to remove DNA lesions during replication (Table 1.2).

Base Excision Repair (BER)

BER is initiated by DNA glycosylases, a group of enzymes which recognize different modified DNA bases. Several DNA glycosylases are found in human cells to recognize different modified bases, including 8-oxoGua DNA glycosylase 1 (hOGG1), hNTH1, hNEIL1, hNEIL2, hNEIL3, hMYH and hSMUG1(Ide & Kotera, 2004; Loft *et al.*, 2006). The hOGG1 is the human homologue of *E.coli* formamidopyrimidine glycosylase (Fpg), and both recognize 8-oxoGua. DNA glycosylases excise lesions by cleavage of the N-glycosylic bond, the link between base and sugar moiety. After the damaged base is removed, an abasic site, which can be either an apurinic or apyrimidinic (AP) site is formed. AP site is removed by the enzyme AP endonuclease in long patch repair or by the enzyme AP lyase in short patch repair. The long patch pathway is involved in repair of spontaneous base

loss, whereas the short patch pathway is involved in repair of oxidized bases. A deoxyribosephosphate residue remains and is removed by phosphodiesterase. The resulting gap is filled by DNA polymerase and finally sealed by DNA ligase (Christmann *et al.*, 2003; Maynard *et al.*, 2009).

Nucleotide Excision Repair (NER)

NER is responsible to remove bulky DNA lesions of ~30 nucleotides in length. CPDs and 6-4 PPs are also repaired by the NER pathway (Cleaver & Crowley, 2002; Sinha & Häder, 2002). NER exists in two forms: global genomic repair and transcription-coupled repair. Global genomic repair is responsible to repair non-transcribed parts, whereas the transcription-coupled repair is responsible to repair transcribed parts of the genome (Zhang *et al.*, 2009). Basically, the mechanism of NER is similar to BER, both involve recognition of DNA lesion, excision of lesion, filling of the gap by DNA polymerase and finally sealing of the nick by DNA ligase. The difference between BER and NER is in the proteins involved. In NER, the DNA lesion is recognized by a group of proteins called Xeroderma pigmentosum complementation group C (XPC), and the DNA lesion is excised by Xeroderma pigmentosum complementation group F (XPF) (Nouspikel, 2009).

Mismatch Repair (MMR)

BER and NER are responsible for correcting DNA lesions before replication, while MMR corrects DNA lesions induced during replication (Christmann *et al.*, 2003). In MMR, the protein MutS binds to the mismatched base. Another protein, MutL, is then attached to the lesion/MutS complex, activating the enzyme, MutH. Activated MutH cleaves at the site of the mismatch bases, and the wrong base is then removed by exonuclease. Finally, the gap is filled by DNA polymerase and sealed by DNA ligase (Li, 2008).

Table 1.2. Summary of DNA repair pathways

Repair pathway	Agent causing damage	Type of lesion
BER	ROS, alkylating agents	Oxidatively modified bases, AP sites
NER	UV	Bulky DNA adducts
MMR	Replication error	Mismatch bases

Effects Of DNA Damage With Focus On Oxidative Stress

As previously mentioned, human cells have various mechanisms to restore and protect DNA. However, even though DNA repair systems are highly effective, in some circumstances they may be inadequate, such as in increased oxidative DNA damage and under stress of carcinogenic substances. It is speculated that oxidative modified DNA damage escapes from repair system and accumulates with age. However, it could also a result of inadequate intake of antioxidant rich foods by older people (Krajcovicová-Kudlácková *et al.,* 2008).

The consequence of oxidatively modified DNA is dependent on the severity, type and site of the modification. Consequences include mutation, with changed phenotypic expression, and apoptosis (Gorbunova *et al.*, 2007). But there may be no apparent effect if the change is in a non-transcribed or inactive region. If oxidative DNA damage occurs in the mitochondria, energy production and the communication between mitochondria and nucleus will be affected (Halliwell, 2001). It must be stressed that the relationship between DNA damage and cancer is currently unclear. However, DNA integrity is central to the wellbeing of the cell and to maintenance of homeostasis and health of the individual (Halliwell, 2007). If oxidatively modified DNA is at key genetic sites such as tumour suppressor genes, proto-oncogenes and those controlling cell proliferation and DNA repair, the likelihood of cancer may be increased.

Apoptosis

Apoptosis (programmed cell death) has been defined as a physiological process of cell deletion that functions as an essential mechanism of normal tissue development. Apoptosis also can act as a protective mechanism to prevent mutant genes passing to the daughter cell, and can be triggered by, among other things, high levels of p53 protein, the product of the *p53* tumour suppressor gene (Hollander & Fornace, 2002). *p53* is activated by DNA damage, and its product arrest the cell cycle at G1 phase until DNA damage is repaired. If DNA damage is irreparable, the level of p53 protein will be maintained at a high level which can permanently arrest the cell cycle at G1 phase (May & May, 1999). Furthermore, a high level of p53

protein can trigger transcription of apoptosis promoting protein bax and initiate apoptosis (Hollander & Fornace, 2002).

Apoptosis can also be triggered by Poly (ADP-ribose) polymerase-1 (PARP-1), which is usually involved in DNA repair. PARP-1 is activated either by oxidation or non-oxidation induced DNA damage. PARP-1 can break down nicotinamide adenine dinucleotide (NAD^+) to nicotinamide and ADP-ribose. PARP-1 then binds with ADP-ribose, and this complex binds DNA repair protein that promotes DNA repair. Activation of PARP-1 favours DNA repair in conditions of mild DNA damage. However, severe DNA damage can activate PARP-induced apoptosis, since under this condition PARP-1 over depletes NAD^+, and resynthesis of NAD^+ requires ATP. Cell death is the result of exhaustion of two key "fuels" in the cell, NAD^+ and ATP (McGowan *et al.*, 1996; Virág, 2005).

Mitochondrial DNA Damage

When compared with nuclear DNA, mitochondrial DNA is under more marked oxidative challenge since ROS formation is mainly as a result of electron leakage from the electron transport chain (ETC). Furthermore, mitochondrial DNA is unprotected by histones and lacks efficient DNA repair enzymes (Lenaz, 2001). Mitochondrial DNA encodes gene products necessary for the electron transport chain such as Complex I, III and IV, and gene products essential for communication with the nucleus (López-Lluch *et al.,* 2008). If these genes are under attack, energy production and cellular functions will become compromised. The degeneration

effects are more notable in non-proliferating cells, such as skeletal muscle and neuronal tissues, and increase with age. Atrophy of muscle and neuronal tissue results from, among other things, ETC deterioration with ATP depletion and increased ROS (Halliwell, 2001). Muscle mass is ~ 40 % lower in humans by the age of ~80 years compared with young adult muscle mass, and brain function declines with age (Halliwell, 2001; Gruber *et al.*, 2008).

Mutation And Carcinogenesis

Carcinogenesis is the making of cancer, a disease characterised by uncontrolled growth and proliferation of abnormal cells. Carcinogenesis is a multistep process involving changes in DNA of key genes. These genes are involved in cell growth and differentiation. Inactivation of tumour suppressor genes, such as *p53*, and activation of tumour promoter genes (change from 'proto-oncogene' to 'oncogene' form) are significant steps in the carcinogenic process. Inactivation or inhibition of DNA repair genes is also very important (Bergoglio & Magnaldo, 2006; Feng *et al.*, 2006).

Ideally, in a dividing cell, all DNA damage is repaired before replication. If DNA damage escapes from the repair system for any reason, a mutation can then be transmitted to the daughter cells and become part of the cell 'lineage' (Gorbunova *et al.*, 2007). Some DNA change is minor and does not trigger cell death or immune response. However, such DNA changes may accumulate with time. As noted, this can lead to cell death. In mitotic tissue no atrophy will result, but such changes can

be carcinogenic if key genes are involved and the damage is not repaired (Figure 1.4).

It is well known that 8-oxodG results in CG to AT transition mutation (Collins, 2004; Cooke *et al.*, 2005). Guanine normally base pairs with cytosine during replication, but 8-oxodG tends to base pair with adenine. Therefore, after replication, the previous G-C base pair is replaced by an A-T base pair in a permanent mutation (Cooke *et al.*, 2003). Due to its high content of guanine bases, the *p53* tumour suppressor gene is a hot spot for G:C → A:T transition mutation (Halliwell, 2000; Peoples & Karnes, 2005). Accumulation of such oxidation-induced mutations in *p53* gene may result in loss of its transcriptional activation, which prevents p53 protein-induced cell cycle arrest and DNA repair, thereby allowing defective genetic information to pass to daughter cells, increasing risk of cancer (May & May, 1999). Currently the relationship between oxidative DNA damage and cancer development is not fully understood. However, studies show the level of 8-oxodG is increased significantly in several types of cancer (Table 1.3).

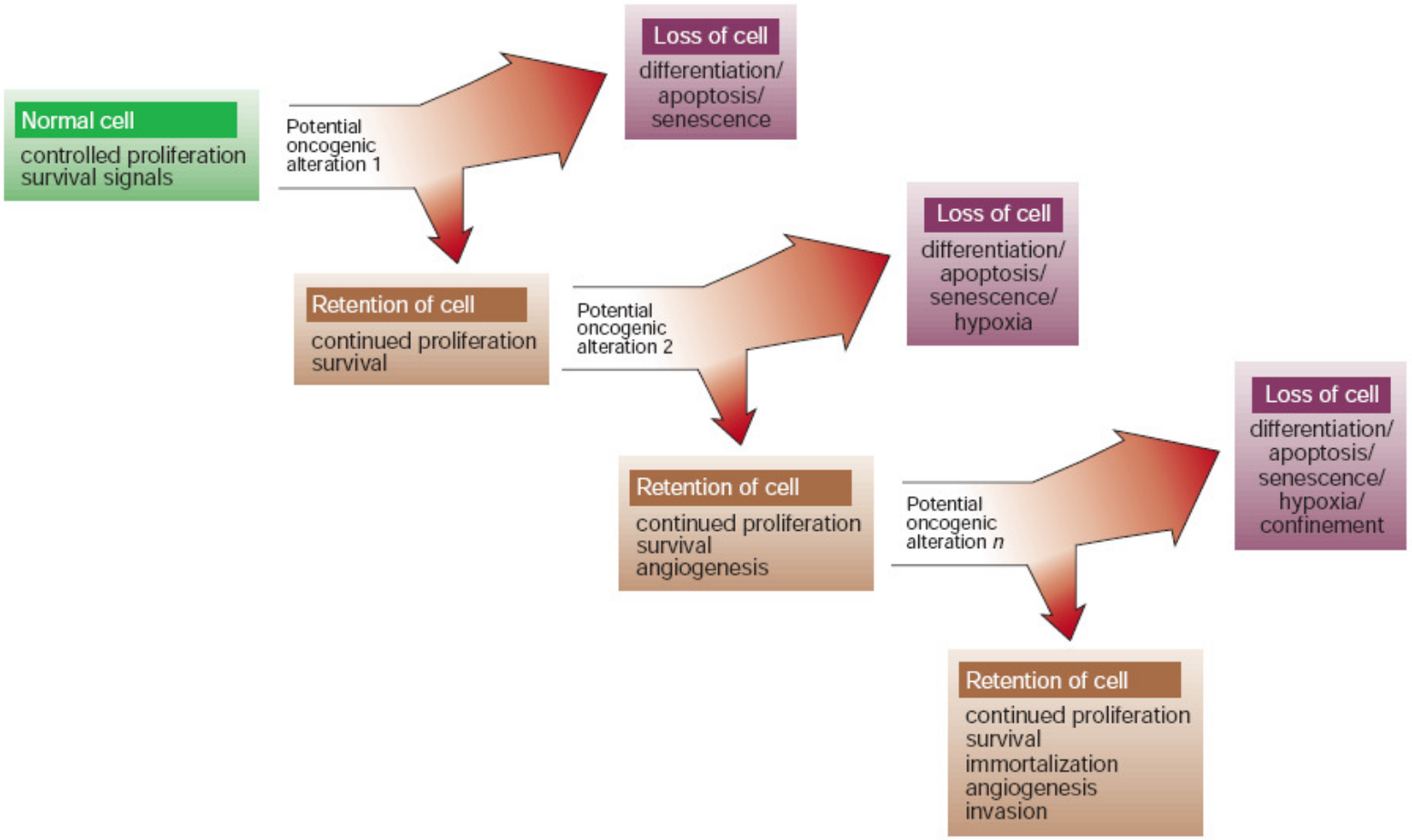


Figure 1.4. Mechanism of carcinogenesis.
(Adapted from Figure 1 in Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature 2001; 411, 342-3.)

Table 1.3. Summary of the reported changes in 8-oxodG in various types of cancer.

Site of cancer	Sample size	Method for 8-oxodG detection	8-oxodG measured in:	Conclusion	Reference
Breast	Control group: 20 females; Cancer group: 49 females	ELISA	Serum	Level of 8-oxodG was ~1.67 times higher in cancer group ($p<0.001$).	(Himmetoglu *et al.*, 2009)
Breast	47 females, and non-cancerous breast tissues from same patients were used as control	HPLC-ECD	DNA of breast tissues	Level of 8-oxodG was ~1.54 times higher in cancer group ($p<0.0001$).	(Matsui *et al.*, 2000)
Cervix	Control: 13 LGSIL: 12 HGSIL: 11	Immunoperoxidase detection	DNA of cervical cells	Level of 8-oxodG was ~1.28 times higher in LGSIL ($p=0.1$). Level of 8-oxodG was ~1.83 times higher in HGSIL ($p<0.001$).	(Romano *et al.*, 2000)

Lung	Control group:48; Cancer group: 46	32p-postlabelling	DNA of lymphocytes	Level of 8-oxodG was ~1.59 times higher in cancer group ($p<0.05$).	(Vulimiri *et al.*, 2000)
Liver	Control group: 82; Cancer group: 36	Immunohistochemical staining	DNA of hepatocytes	Level of 8-oxodG was ~1.56 times higher in cancer group ($p<0.0001$).	(Tanaka *et al.*, 2008)
Haematological diseases	Control group: 10; Cancer group (total 44) comprising: Malignant lymphoma: 13 ATL: 11 AML: 7 ALL: 3 MDS: 10	ELISA	Urine	Level of 8-oxodG was ~2.13 times higher in ATL group ($p<0.005$). Level of 8-oxodG was elevated in all other groups, however, this was not statistically significant.	(Honda *et al.*, 2000)

HPLC-ECD: High performance liquid chromatography-Electrochemical Detector
ELISA: Enzyme-linked immunosorbant assay
LGSIL: low grade squamous intraepithelial lesion
HGSIL: high grade squamous intraepithelial lesion
ATL: T cell leukaemia:lymphoma
AML: acute myeloid leukaemia
ALL: lymphoid leukaemia
MDS: myelodysplastic syndrome

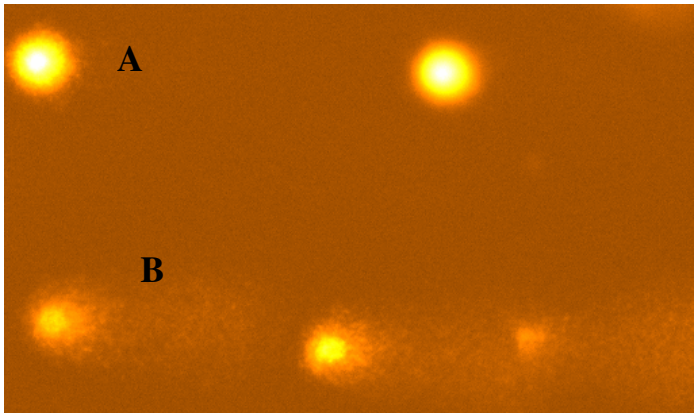
Assessing DNA Damage And Repair

Cancer is a multifactorial disease, but damaging (mutagenic) changes to DNA are crucial. A number of methods for measuring DNA damage have been developed. The most commonly used methods for measuring DNA damage include the comet assay and liquid chromatography- tandem mass spectrometry (LC-MS/MS) (Cooke *et al.*, 2008). Other methods include cytokinesis-block micronucleus assay (Mateuca *et al.*, 2006), and alkaline unwinding of DNA (Guetens *et al.*, 2002). These tests do not probe for specific mutations, but measure DNA damage in more general or 'global' terms. Therefore, these are not methods that can be used to diagnose cancer or directly assess cancer risk. Nonetheless, if 'global' DNA damage is increased it is likely that key genes related to cancer may be affected. Furthermore, increased DNA damage is likely to be detrimental to the cell in other ways not directly related to cancer. Therefore, measurement of DNA damage has become widely adopted in biomonitoring studies and in the study of therapeutic and lifestyle effects on health (Pereira *et al.,* 2010; Cavallo *et al.*, 2009; Sardas *et al.*, 2009).

The Comet Assay

In the comet assay, nucleated cells are embedded in low melting point agarose on a microscope slide, and the membrane and histones are removed by high salt solution (Singh *et al.*, 1988; Wong *et al.*, 2005; Collins *et al.*, 2008). DNA is generally organized in a tightly supercoiled form, but supercoils relax around areas of DNA breaks, unwinding and spilling out as a 'halo' surrounding the nucleoid

(Shaposhnikov *et al.*, 2008). Electrophoresis at neutral, mildly alkaline or strongly alkaline conditions follows the unwinding step (Collins, 2004; Møller, 2006). DNA contains phosphate groups that are negatively charged at alkaline pH, and so relaxed loops of damaged DNA containing breaks are pulled towards the anode during electrophoresis, forming a comet 'tail', while the DNA remaining coiled within the nucleoid forms the comet 'head' (Figure 1.5) (Tice *et al.*, 2000). The comet is visualized by a DNA staining fluorescent dye or silver stain (Tice *et al.*, 2000; Shaposhnikov *et al.*, 2009). The most frequently used stains are ethidium bromide, propidium iodide, 4,6-diamidino- 2-phenylindole (DAPI) and YOYO-1 (Collins, 2004). DNA damage is then scored using either visual or computerized image analysis.



A, Typical undamaged nucleoid

B, Typical damaged nucleoid

Figure 1.5. Typical undamaged and damaged nucleoids after running the comet assay.

In order to achieve various objectives, various modifications of the comet assay have been developed (Table 1.4 and described in more detail below). These allow us to look at the *in vitro* effects and explore mechanisms of action of

potentially genotoxic or genoprotective agents (Azqueta *et al.*, 2009; Cemeli *et al.*, 2009).The comet assay can be applied to nearly all eukaryotic cells (red blood cells cannot be used as they lack a nucleus), but lymphocytes are the most commonly used type of cell in human studies. Frozen lymphocytes are also suitable for the comet assay, with the exception of the timed repair model of comet assay (Duthie *et al.*, 2002).

Under controlled conditions, cells can be incubated with a test agent or somehow stressed *in vitro,* and the resulting DNA damage in the treated cells can then be measured using the comet assay. Of more interest perhaps is the effect of a 'challenge' to DNA after the cells have been exposed to a putative protective agent. This approach is widely used in determining the potential genoprotective effects of dietary antioxidants, herbs and phytochemicals (Duthie & Dobson, 1999; Johnson & Loo, 2000; Szeto & Benzie, 2006). The comet assay can also be used as an *'in vivo'* biomonitoring tool for investigating the effects of foods, food components, or supplements that are believed to have a genoprotective effect (Collins *et al.*, 2003; Møller *et al.*, 2004). In this model, lymphocytes or possibly other cells are harvested from blood or tissues and the comet assay run to detect changes in baseline damage or in resistance to an *in vitro* stress.

Table 1.4. Overview of applications of the comet assay

Application	Comet version
Assess intrinsic or treatment-related resistance of cellular DNA to oxidative stress	Cells with or without a standardized oxidant challenge
Genotoxic effects of treatment/test agents	Incubate cells with testing substance, followed by neutral/alkaline/enzyme-linked comet assay.
Background level of oxidative DNA damage	Enzyme-linked comet assay. Most commonly used enzymes are Endo III (oxidized pyrimidines) and FPG (oxidized purines).
DNA repair	a) Cells are treated with DNA damaging agent *in vitro* and then incubated in culture medium. Cells are analyzed with the comet assay at certain time point to assess repair. b) Cells are embedded in agarose gel before treatment with DNA damaging agent, followed by incubation in culture medium. Cells are analyzed with the comet assay at certain time point. c) Cultured cells are stressed and then incubated with cell extract from cell of interest to assess effects of cell extract constituents on rate or extent of repair of damaged cells

Neutral Comet Assay

The comet assay was first introduced by Ostling and Johanson (1984), who used what is termed a 'neutral' pH of 9.5 for lysis and electrophoresis (Ostling & Johanson, 1984). This pH is below the limit for DNA unwinding, and was reported by Singh (1988) to detect only DSB, with more strongly alkaline conditions (pH 10 or above) needed for unwinding and detection of SSB (Singh *et al.*, 1988). However, SSBs are detected in the neutral or mildly alkaline comet assay, which has been shown to have the same limit of detection of DNA damage as the alkaline comet assay (Collins, 2004), although the use of neutral pH does affect the comet image

obtained, as the comet tails are less pronounced, and this lessens the sensitivity, or DNA damage score gradient, of the assay (Angelis *et al.*, 1999; Collins, 2004). This can be an advantage when a less sensitive method is needed, for example when investigating cells that have a large amount of background or induced damage is high (Angelis *et al.*, 1999).

Alkaline Comet Assay

Singh and co-workers (1988) presented the alkaline version of the comet assay, in which DNA is allowed to unwind at pH >13 (Singh *et al.*, 1988). The strongly alkaline conditions make for clearer images, and a steeper gradient of response. Besides SSBs, other types of DNA damage, such as alkaline labile sites (ALS), can be detected at strongly alkaline conditions (Tice *et al.*, 2000; Collins, 2004). SSBs are formed from alkali-labile sites at pH >13, revealing otherwise hidden damage. Employing milder alkaline (pH=12.3) conditions prevents conversion of alkaline labile site into breaks. Therefore, by modifying the pH of the lysis/unwinding and/or electrophoresis steps over the range 9.5-13.5, the comet assay models of different sensitivity (but of similar limits of detection) can be applied (Angelis *et al.*, 1999; Collins, 2004).

Enzyme-Linked Comet Assay

The use of enzymes can produce a comet assay model of greater sensitivity and, in addition, a more specific assay can be developed. In addition to SSBs, DSBs and AP sites, other types of damage, such as oxidized bases or UV-induced dimers,

which do not cause strand breaks, exist. These types of DNA damage cannot be detected unless lesion-specific enzymes are added (at the post-lysis stage) to create breaks at the sites of damage. Enzymes that have been used to date include: Endonuclease III (Endo III), which detects oxidized pyrimidines; Fpg which detects oxidized purines and ring-opened purines (Azqueta *et al.,* 2009; Gaivão *et al.,* 2009); T4 endonuclease V (T4EV) which detects UV-induced CPDs (Decome *et al.*, 2005); methyladenine DNA glycosylase II (AlkA) that reveals 3-methyladenine sites (Collins *et al.*, 2001a); uracil DNA glycosylase (UDG), which exposes sites of mis-incorporated uracil (Kapiszewska *et al.*, 2005).

Lymphocytes Extract Comet Assay

In 2001, Collins and co-workers developed a version of the comet assay to measure DNA repair, rather than damage. DNA repair capacity is determined by incubating a cell extract with substrate cells whose DNA contains specific, induced lesions (Collins *et al.*, 2001b). The cell extract is prepared from lymphocytes pre-treated *in vitro* or '*in vivo'* (by for example supplementation or exercise) and contains DNA repair enzymes. This version of the comet assay can determine either NER or BER by using substrate cells with different types of DNA lesions (Gaivão *et al.,* 2009). For BER assessment, 8-oxoGua is introduced to HeLa cells (substrate cells) by use of the photosensitizer Ro 19-9022 plus visible light (Collins *et al.*, 2001b). Substrate HeLa cells are embedded in low melting point agarose on a microscope slide and lysed as usual. The DNA of the HeLa cells is then exposed to the lymphocyte extract. The repair enzyme hOGG1 in the cell extract will excise

damaged nucleotides and create strand breaks at areas of damage (Collins *et al.*, 2003). After running the comet assay, a greater amount of damage will be seen in those HeLa cells exposed to cell extracts that contain higher hOGG1 levels. Therefore, if an intervention increases repair capacity in cells by increasing hOGG1 activity, more DNA damage will be seen in the substrate cells (Collins *et al.*, 2001b; Collins *et al.*, 2003). For NER assessment, the basic principle and procedure are same as BER. However, a different type of damage is repaired by NER (CPDs and 6-4 PPs), and so UVB or UVC instead of Ro 19-9022 is used to induce damage in substrate cells (Gaivão *et al.*, 2009)

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS): A Specific And Sensitive Tool For Damaged Nucleotide Bases

The LC-MS/MS is a very sensitive and selective hyphenated instrument which demonstrates the separation ability of liquid chromatography and identification and quantitation ability of mass spectrometry. The hyphenated feature makes LC-MS/MS able to detect, identify and measure chemical compounds in complex mixtures in a very specific and sensitive way. This technique can be used to identify and measure different types of compounds, and due to its sensitivity and specificity it is a valuable tool in measuring oxidized or otherwise damaged nucleotide bases. The basic components and principles of LC-MS/MS are presented briefly below.

High Performance Liquid Chromatography (HPLC)

Liquid chromatography refers to any chromatographic technique where the mobile phase is liquid (Meyer, 2004). The separation is dependent on the different equilibrium constants of components between mobile phase and stationary phase (Meyer, 2004). The classification of LC is based on the nature of the stationary phase and the process involved, which include,

1. Adsorption chromatography, the separation is based on the competition between liquid mobile phase and solid stationary phase.
2. Partition chromatography, the separation is based on the competition between liquid mobile phase and inert solid support liquid stationary phase.
3. Size exclusion, the separation is based on size. The stationary phase is made up by various sizes of particle.
4. Ion exchange, the separation is based on the competition between liquid mobile phase and charged solid stationary phase.

HPLC generally employs partition chromatography and separate constituents within a mixture based on polarity differences under high pressure within an analytical separation column. The most generally used analytical columns in HPLC are i) Normal phase, in which the stationary phase (the column packing material) is more polar than the mobile phase, and ii) Reversed phase, in which the stationary phase is less polar than the mobile phase. The column separation ability is

dependent on the particle size of the packing material and column length. Usually a column with smaller particle size and longer length has greater separation ability. HPLC can detect a wide variety of compounds when coupled with different types of detectors, the most commonly used including UV/Visible detector, photodiode array (PDA) detector, fluorescence detector, electrochemical detector (ECD) and MS. Among these detectors, MS is the most powerful and is described below.

Mass Spectrometry (MS)

MS is a very powerful analytical technique which can be used to identify unknown molecules, determine molecular mass and composition, isotopic distribution and chemical properties of molecules (Downard, 2004). It can also be used to quantitate known molecules in a sample. An MS can be considered as an instrument which separates ions generated from molecules or atoms based on the mass to charge ratio (*m/z*) of the ions (Downard, 2004). An MS comprises an ion source, a mass analyzer and an ion detector (Figure 1.6).The ion source is used to fragment/ ionize analytes, and various ionization methods can be used according to analyte mass and polarity. Generated ions are separated by the mass analyzer using a magnetic or electric field. The ion detector can create and magnify a current when ions strike it (Gross, 2004). With the exception of some ionization methods which may operate at atmospheric pressure, an MS usually operates at low pressure or in a vacuum in order to minimize unwanted collisions between ions and gas molecules. After detection, information is passed to the data system for data handling and output (Downard, 2004; Gross, 2004).

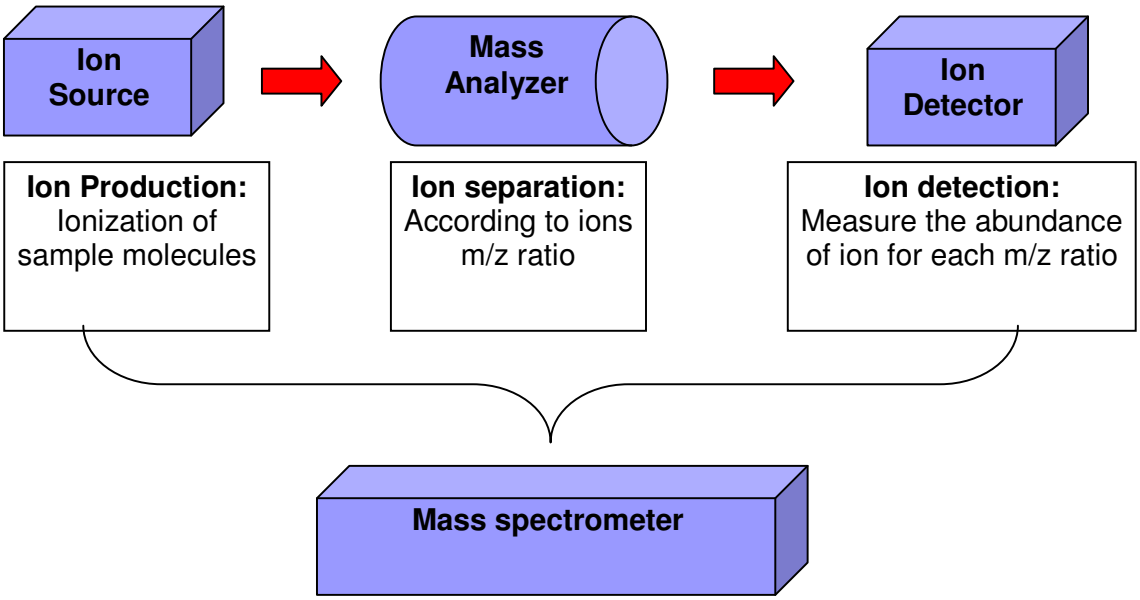


Figure 1.6 Components of a mass spectrometer.

Various mass analyzers are available which include quadrupoles, time-of flight mass spectrometer, fourier transform mass spectrometer and isotope mass spectrometer (Downard, 2004). Tandem mass spectrometry (MS/MS) approach is also available. The MS/MS is any spectrometer which contains two or more mass analyzers. Figure 1.7 shows the principle of MS/MS. The first mass analyzer is used to separate parent ions of interest after 'first round' ionization. Selected parent ions are then passed to the dissociation chamber to undergo fragmentation. Collision induced dissociation (CID) is the most typical method used to induce fragmentation (Gross, 2004). In CID, the selected parent ions are collided with neutral gas molecules. During collision some kinetic energy is changed to internal energy and initiates fragmentation into daughter ions. The second mass analyzer is used to analyze these fragmented ions (Matern & Magera, 2001). The selection and

dissociation step can be repeated for further fragmentation in an 'MS^n analyzer'. Theoretically, the amount of structural information that can be obtained is increased with the number of MS analyzers. The ability of separating parent ions of interest makes MS/MS technique useful to study a particular molecule within a mixture. One example is in measurement of 8-oxodG in urine matrix.

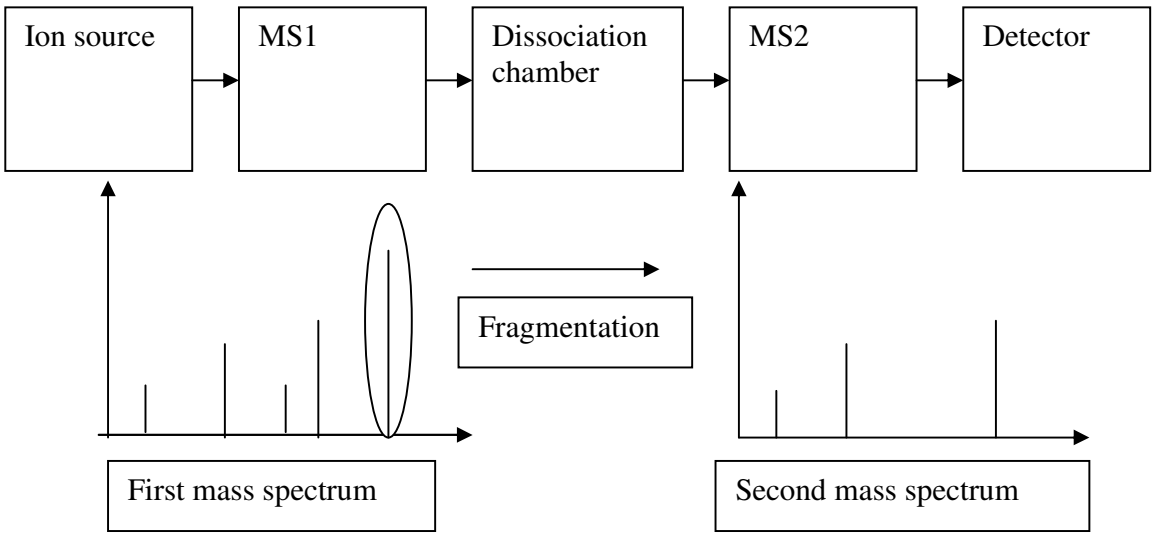


Figure 1.7. Principle of MS/MS.

Application Of LC-MS/MS For 8-oxodG

Our research group has developed a novel LC-MS/MS method, which does not involve extensive sample pre-treatment with solid phase extraction (SPE) and simply requires sonication and centrifugation of urine before injection (Lee *et al.*, 2010) .

Formerly, urinary 8-oxodG has been regarded as a biomarker of oxidatively damaged DNA. Recently, Cooke *et al.* suggest the origin of urinary 8-oxodG is probably not from repair of oxidatively damaged guanine bases in DNA, but from sanitation of oxidized deoxyguanosine triphosphate (dGTP) or diphosphate (dGDP) in the nucleotide pool (Cooke *et al.*, 2008). Therefore, urine 8-oxodG has been proposed as a biomarker of 'whole body' oxidative stress, rather than a biomarker representing oxidatively DNA damage. This is how it was used in this study.

Cytokinesis-Block Micronucleus Assay (CBMN)

Micronuclei, also known as Howell-Jolly bodies, are acentric chromosome fragments (lacking a centromere) or whole chromosomes which cannot attach to spindle poles at metaphase during cell division (mitosis or meiosis) (Mateuca *et al.*, 2006). At telophase, a nuclear envelope will form, surrounding the nucleus of the daughter cell. However, acentric chromosome and unattached whole chromosomes will not be included in the main nucleus, and this nuclear material becomes surrounded by another membrane, forming a 'micronucleus' (Mateuca *et al.*, 2006). Micronuclei indicate chromosome damage in cells.

Micronuclei form in dividing cells only, therefore, assessing the frequency of micronuclei is most accurate in dividing cells. The CBMN is based on allowing cells to complete one nuclear division only, by using cytochalasin –B (CYT-B) (Norppa & Falck, 2003; Mateuca *et al.*, 2006). CYT-B allows accumulation of dividing cells in the binucleated state due to CYT-B inhibiting actin polymerization, which is essential for the microfilament formation, and is an essential component to separate the dividing cells. Therefore, CYT-B treatment allows accumulation of cells at the binucleated state, without interference by the proportion of cells at the dividing state (Norppa & Falck, 2003). The ratio of micronucleated binucleated cell/ binucleated cells provides a reliable index of chromosome loss and breakage. However, this assay can be applied to dividing eukaryotic cells only. This test was not used in this study and will not be mentioned further.

Alkaline Unwinding

Alkaline unwinding is a technique for detecting DNA breaks in whole cell population. It is based on facilitating DNA unwinding under alkaline conditions (Guetens *et al.*, 2002). Cells are lysed by detergent at high salt concentration. DNA is then allowed to unwind at moderate alkaline conditions (pH 12.3-12.4). DNA unwinding step is a time-dependent process at moderate alkaline pH (Baumstark-Khan, 1994) and is stopped by neutralization after a certain period of incubation time. Moderate alkaline conditions allow removal of histone protein, inactivation of DNA-degradative enzymes and unwinding of the double-strand DNA.

DNA under alkaline conditions will start unwinding at the broken point and from the ends of DNA. Types of DNA damage such as SSB, DSB, ALS and depurinisation and depyrimidation DNA, will act as the starting point of unwinding. After a certain period of time, the unwinding process is stopped by neutralization (Guetens *et al.*, 2002). Single-strand DNA and double-strand DNA are then separated by hydroxyapatite column. The eluate is incubated with a DNA binding stain, for example Hoechst 33258 stain. The number of strand breaks can be calculated from the amount of single-strand DNA and double-strand DNA. The alkaline unwinding step is affected by many factors, such as temperature, alkaline strength, and duration (Guetens *et al.*, 2002). If the testing substances are inhibitors of topoisomerases, the degree of unwinding will be decreased. Therefore, the testing condition must be controlled in order to obtain reliable results. This method was not used in this study and will not be mentioned further.

Summary: DNA Damage And Repair Testing

In summary, maintenance of DNA integrity through protection and repair is crucial to the maintenance of the health of the cell. The comet assay is a versatile, reliable and sensitive test of DNA damage and repair. 8-oxodG can be measured very specifically and sensitively using LC-MS/MS and has been used quite extensively to assess DNA damage, although in urine it most likely is a marker of oxidative stress, rather than a biomarker of DNA damage *per se*. Both these tests have been used to investigate the effects of disease, age, and lifestyle changes on DNA damage. Of interest in this study is the effect of dietary changes, or supplementation, on oxidation-induced DNA damage. Of particular interest is the effect of natural products that contain antioxidants.

Antioxidants: Types And Sources

The human body is constantly under oxidative challenge by different types of ROS. In order to protect vital biological molecules, the body has evolved various antioxidant defence systems to protect against ROS action (Collins, 1999; Benzie, 2000). An antioxidant is defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell & Gutteridge, 2007). Antioxidants can be endogenous or exogenous (Benzie, 2003). Endogenous antioxidants are synthesized within the human body. Exogenous antioxidants are those that are required to be obtained from the diet.

Mechanisms of antioxidant protection against oxidative damage include prevention of ROS formation, destruction or inactivation of formed ROS and breaking of ROS chain reactions (Benzie, 2000; Benzie, 2003). These mechanisms are summarized in Table 1.5. The human antioxidant system is very complex. Different parts of the body have various antioxidants, and have various mechanisms to inter-relate with each other in a coordinated overall antioxidant system.

Table 1.5. Summary of antioxidant protective mechanisms

Mechanism	Example
Prevention of ROS formation	Metal ions binding protein, such as transferrin, ferritin (iron) and caeruloplasmin (copper)
Enzymatic reaction for destruction ROS	Superoxide dismutase, glutathione peroxidase and catalase.
Quenching to absorb energy of ROS	Carotenoids
Scavenging to directly interact with ROS and prevent interaction with important biomolecules	Uric acid, bilirubin, glutathione, Vitamin C (ascorbic acid) and α-tocopherol (and possibly other 'vitamin E' isomers)

Enzymatic Systems

Several enzymes act as antioxidants within (mainly) cells. These enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (Benzie, 2000). There are three forms of SOD in humans, including cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD and extracellular SOD. SOD can dismutate superoxide ($O_2\bullet^-$) into H_2O_2 (McCord & Edeas, 2005). When free iron or copper ions are present, H_2O_2 can form the highly reactive ROS, •OH. Therefore, to avoid the buildup of H_2O_2, high levels of GPx and catalase are found in areas where H_2O_2

is produced. GPx can remove H_2O_2 by oxidizing gluatathione (GSH) to glutathione disulfide (GSSG) and catalase can convert H_2O_2 to oxygen and water directly (Matés, 2000). The co-operation and chemical equations of these enzymatic reactions are summarized in Figure 1.8 and Table 1.6.

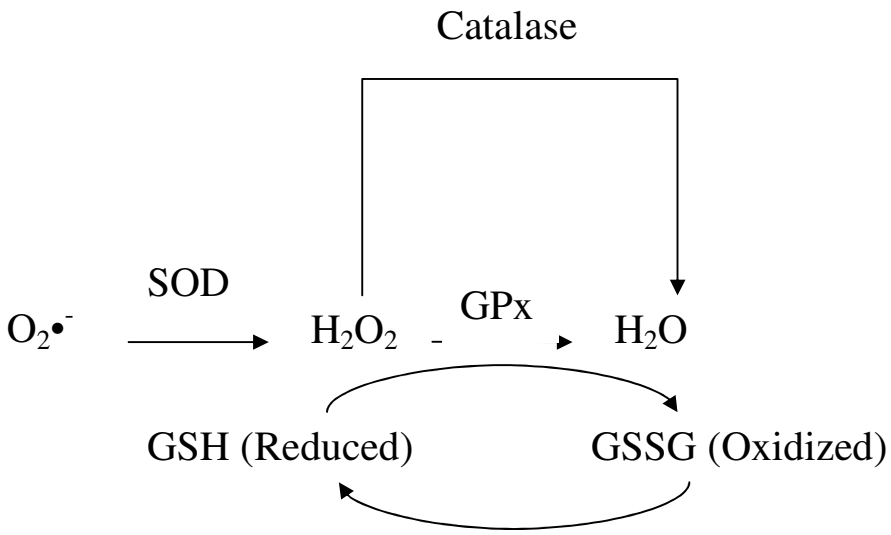


Figure 1.8. Outline of co-operative action of antioxidant enzymes in human body

Metal Ion Binding Proteins

Within the human body, even though H_2O_2 is continuously produced and can be converted to •OH, this occurs at a very slow rate unless transition metal ions are present. Free iron or copper ions can act as electron donors and accelerate •OH formation by the Haber-Weiss / Fenton reaction (Halliwell, 2009). Therefore, the body has metal ion binding proteins, transferrin and ferritin, for binding iron, and caeruloplasmin for binding copper and this is regarded as an antioxidant action

(Table 1.5) (Benzie, 2000). The chemical equations of Haber-Weiss / Fenton reaction are summarized below.

Chemical equations of Haber-weiss / Fenton reaction:

Fe^{3+} (or Cu^{2+}) + $O_2\bullet^-$ ⟶ Fe^{2+} (or Cu^{+}) + O_2

Fe^{2+} (or Cu^{+}) + H_2O_2 ⟶ Fe^{3+} (or Cu^{2+}) + •OH + OH^-

Net equation:

$O_2\bullet^- + H_2O_2 \xrightarrow[\text{Metal ion catalyst (Cu or Fe)}]{} \bullet OH + OH^- + O_2$

Table 1.6. Summary of location and reaction of endogenous antioxidants

Endogenous antioxidant	Location	Reaction
SOD	Nucleus, Cytosol, Peroxisomes, Mitochondria	$2O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2$
GPx	Nucleus, Mitochondria	$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$
Catalase	Peroxisomes	$2H_2O_2 \rightarrow 2H_2O + O_2$
Transferrin	Plasma	Ferric ions (Fe^{3+}) storage (In redox inactive form)
Ferritin	Cytosol	Ferric ions (Fe^{3+}) storage (In redox inactive form)
Caeruloplasmin	Plasma	Copper ions storage (In redox inactive form)

Scavenging And Quenching Antioxidants

Uric acid, bilirubin, glutathione, ascorbic acid and flavonoids are scavenging antioxidants, and β-carotene is a quenching antioxidant (Benzie, 2000; Valko *et al.*, 2006). Among the various antioxidants, α- tocopherol is special since it can act as a scavenging and as a quenching antioxidant. Ascorbic acid, β-carotene and α- tocopherol are dietary-derived antioxidants. Uric acid is the result of purine catabolism (Kutzing & Firestein, 2008). At physiological pH almost all uric acid is ionized into urate. Since uric acid is small and water soluble, it can diffuse and dissolve into fluid lining the upper respiratory tract (Halliwell & Gutteridge, 2007). Therefore uric acid is significant in scavenging oxidizing air pollutants, such as ozone (O_3) and nitrogen dioxide (NO_2•). Bilirubin is a product of haemoglobin catabolism. At physiological pH, unconjugated bilirubin is water insoluble and mainly binds to albumin within the plasma, where it can provide antioxidant protection for fatty acids and proteins due to its potent scavenging power against peroxyl radicals and singlet oxygen (Stocker, 2004; Watson, 2009).

GSH is a tripeptide synthesized by γ - glutamylcysteine synthetase and glutathione synthetase, and is a potent scavenger of •OH, HOCl•, HNOO•, RO• and ROO• (Valko *et al.*, 2006). Glutathione also works in conjunction with GPx to degrade peroxides. Oxidized GSH (GSSG) is recycled to the reduced form (GSH) by the action of glutathione reductase (GR), with electrons and hydrogen supplied by NADPH (Halliwell & Gutteridge, 2007).

Plants contain many antioxidants that humans do not manufacture. Apart from ascorbic acid, 'vitamin E' and the carotenoids, there are thousands of phenolic compounds (Duthie *et al.*, 2003). Major classes include flavonols, flavones, flavan-3-ols, flavanones, anthocyanins and isoflavones. Phenolic compounds show powerful antioxidant activities and can scavenge hypochlorous acid (HOCl), hydroxyl (•OH), peroxyl (ROO•) and $O_2^{\bullet -}$ (Halliwell *et al.*, 2005). The antioxidant action is due to the hydrogen atom donating capacity from the aromatic hydroxyl group (Duthie *et al.*, 2003). Phenolic compounds show powerful antioxidant activities *in vitro*, Szmitko & Verma reported that phenolic compounds in red wine can inhibit low-density lipoprotein (LDL) oxidation (Szmitko & Verma, 2005), and our group and others have shown that phenolic rich foods and herbs and tea have high antioxidant content (Benzie *et al.*, 1999; Wachtel-Galor *et al.*, 2004b). However, results from *in vivo* studies are conflicting (Halliwell *et al.*, 2005). It is known that bioavailability of polyphenolics is very low, and it is reported that high doses of polyphenolics, and perhaps other antioxidants, can induce DNA damage *in vitro* (Halliwell *et al.*, 2005). Further study is needed to investigate the physiological effects and importance of phenolic compounds.

Lingzhi, Green Tea, Bilberry And Cordyceps As Sources Of Antioxidant And Other Protective Effect On DNA

Most human endogenous antioxidant systems have a limited ability to be up-regulated. Increased levels of some endogenous antioxidants are often associated

with disease. Related examples include jaundice and gout, which are caused by increase bilirubin and uric acid, respectively (Kutzing & Firestein, 2008; Watson, 2009). A more simple and direct way to enhance antioxidant defence is suggested to be increased intake of dietary antioxidants.

It has been suggested that increased intake of antioxidant-rich fruits, vegetables or herbs can boost the antioxidant defence if enough antioxidants can be absorbed. Evidence shows that oxidative damage is a key factor in ageing and age-related disease, and conceptually at least increased intake of dietary antioxidants may help prevent oxidative damage *in vivo*, help maintain DNA integrity, and so promote healthy ageing. Extensive experimental, supplementation and epidemiological studies of dietary antioxidants and health have been performed, but results are contradictory and do not show clear evidence of benefit (Duthie & Dobson, 1999; Wu *et al.*, 2003; Møller *et al.*, 2004; Wachtel-Galor *et al.*, 2004c; Leung *et al.*, 2006). Many of these have been with 'pure' antioxidants such as vitamin C or E, but a more rewarding approach may be to look at whole foods or extracts of whole foods. Currently, there is great interest in 'functional foods' and nutraceuticals. In Hong Kong (and greater China) there is a long and well respected culture of traditional herbal medicine. Many of the 'herbs' are in fact foods, fungi or teas, and most are taken for general health promotion. Many have high antioxidant content. Most require further scientific study to confirm reputed health benefits, particularly in relation to oxidative stress and DNA protection. We have identified four 'herbs' (two types of fungus, one berry and green tea) that have high reputation

in Chinese culture for health benefit. These are described in more detail below.

Lingzhi

Ganoderma lucidum (Curtis: Fr) P. Karst, also known as Lingzhi in China, belongs to Ganodermaceae or Polyporaceae of Aphyllophorales (Wachtel-Galor *et al.*, 2004a). Lingzhi is a woody mushroom, and has high and longstanding reputation for promoting longevity in Asian countries. Lingzhi contains a wide range of components including polysaccharides, ganoderic acids, proteins, minerals, vitamins and unsaturated fatty acids (Paterson, 2006). Among these, polysaccharides and triterpenes are well known and are believed to be responsible for pharmacological effects, such as the anti-tumour, immunomodulatory, hypoglycaemic and antioxidant properties reported for Lingzhi (Ooi & Liu, 2000; Paterson, 2006).

Polysaccharides

More than 200 polysaccharides have been identified either from the mycelial biomass in the culture or from the fruit body, mycelia and spores of Lingzhi (Paterson, 2006). Polysaccharides from Lingzhi are heteropolysaccharides which contain mannose, fucose, xylose and galactose (Wachtel-Galor *et al.*, 2004a). The basic polysaccharides structure in Lingzhi is β-glucan with different types of glycosidic linkages (Wasser, 2002). The most anti-tumourigenic β-glucan is the one with β-1,3-linkage and containing β-1,6-D-glucosyl branch. Furthermore, the anti-tumour activity increases with molecular weight (Ooi & Liu, 2000). The anti-tumour effect of polysaccharides in Lingzhi is believed to be due to stimulating host

immunity, rather than direct cytotoxicity against tumour cells. Wang and co-workers observed the production of interleukin (IL)-1β, tumour necrosis factor (TNF)-α and IL-6 in monocytes culture which is a human cell line, and this showed a dose response with Lingzhi polysaccharides (Wang *et al.*, 1997). A 12 week supplementation study with 1800 mg/day Lingzhi polysaccharide extract in advanced-stage cancer patients (n = 30) also showed a significant increase in plasma levels of IL-2, IL-6 and interferon (IFN)-δ (Gao *et al.*, 2003). Until now, that the anti-tumour mechanism of polysaccharides is through host mediated immunity effects remains to be confirmed, however, both *in vitro* and *in vivo* studies show Lingzhi polysaccharides can stimulate the production of cytokines.

Triterpenes

The bitter taste of Lingzhi is from triterpenes. Triterpenes is a subclass of terpenes and the chemical structure is based on lanosterol (Figure 1.9) (Wachtel-Galor *et al.*, 2004a). Ganoderic acid A and B were the first two triterpenes identified, and now more than 130 triterpenes have been described (Paterson, 2006). The anti-tumour mechanisms of triterpenes are still under research, but effects are believed to be mediated by inhibition of cell growth and cytotoxicity, rather than due to effects on the host immune system. Lin and co-workers suggested triterpenes-rich extract from Lingzhi mycelia inhibits human hepatoma Huh-7 cells growth by suppressing protein kinase C and arresting G2-phase cell cycle, effects not seen in normal human hepatocytes (Lin *et al.*, 2003). Furthermore, triterpenes demonstrated cytotoxicity against HL-60 cells after 24 hours' incubation (González *et al.,* 2002).

Lingzhi has been reported to have antioxidant properties, and the plasma antioxidant capacity (FRAP value) increased significantly (~ 2-3 %), after a single dose of 3.3g Lingzhi powder and peaked at 90 min post ingestion. This implies that some bioactive compounds in Lingzhi are absorbed by humans and may help with lowering oxidative stress (Wachtel-Galor *et al.*, 2004b).

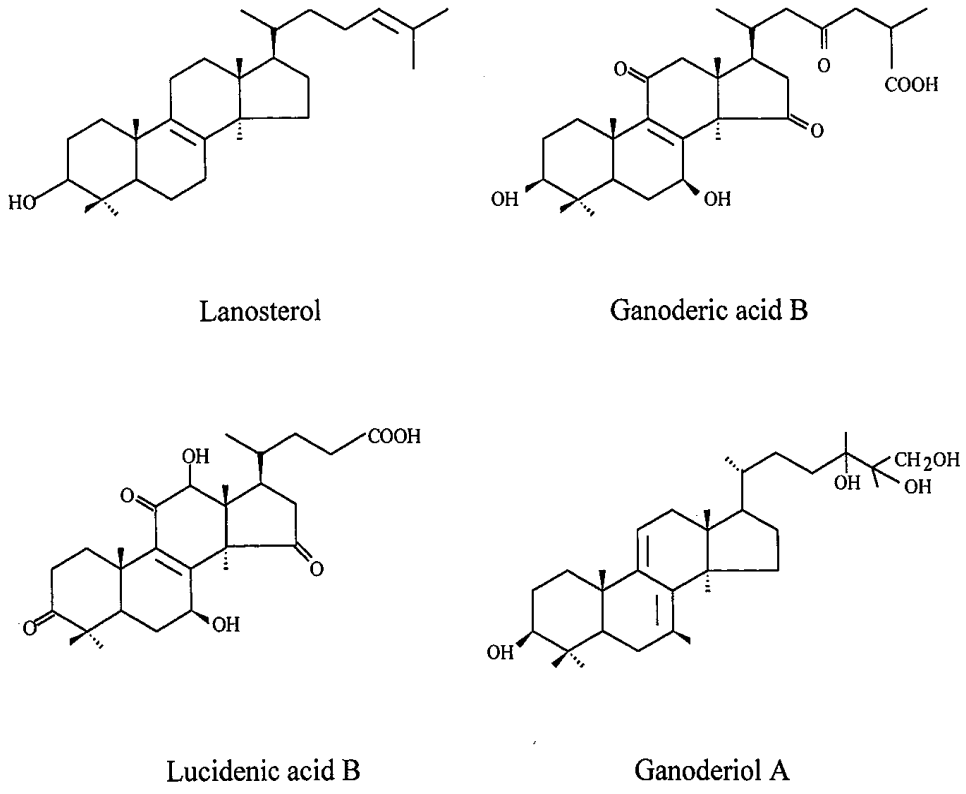


Figure 1.9. Chemical structure of lanosterol and some common triterpenes isolated from Lingzhi.
(Adapted from Figure 2 in Wachtel-Galor S, Buswell JA, Tomlinson B, Benzie IF. Lingzhi polyphorous fungus (Ganoderma lucidum). In Herbal and Traditional Medicine: molecular aspects of health, pp. 179-228 [L Packer, CN Ong and B Halliwell, editors]. New York: Marcel Dekker.)

Green Tea

Tea (*Camellia sinensis*) is the second most consumed beverage worldwide, just next to water (Weisburger & Chung, 2002). There are three main types of tea, which are green tea (unfermented), black tea (fermented) and oolong tea (semi-fermented) (Shukla, 2007). Green tea is mainly consumed in Asian and Middle Eastern countries and accounts for 20 % of tea production (Khan & Mukhtar, 2007). Green tea leaves are steamed or fired immediately once plucked off from the tea plant. The heating process inactivates polyphenol oxidase, the fermentation enzyme, therefore, the composition of green tea is close to fresh leaves (Khan & Mukhtar, 2007). Green tea is rich in minerals and antioxidants. A single cup of 1% w/v green tea can provide ~200 mg of catechins (Yang *et al.*, 2009).

Polyphenols

Catechins are polyphenolic compounds, and are the main components of green tea, accounting for 30-40% of dry weight of tea leaves. The major types of catechins in green tea are epigallocatechin-3-gallate (EGCG) (the major type), epigallocatechin (EGC), epicatechin (EC) and epicatechin-3-gallate (ECG) (Khan & Mukhtar, 2007) (Figure 1.10).

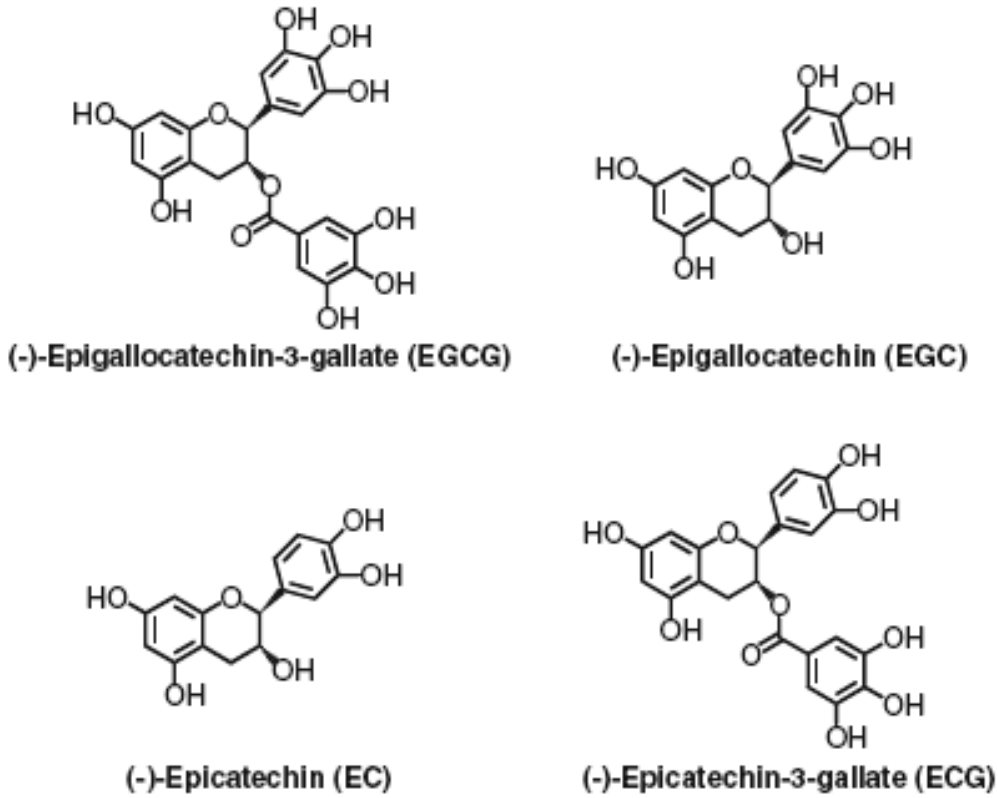


Figure 1.10. Chemical structure of major green tea catechins.
(Adapted from Figure 1 in Yang CS, Lambert JD, Sang S. Antioxidative and anti-carcinogenic activities of tea polyphenols. Arch Toxicol. 2009;83(1):11-21.)

Most human epidemiological studies show green tea consumption has inverse relationship with cancer. Population-based case-control studies demonstrated green tea consumption effectively decreases gastric and oesophageal cancer risk in Chinese population (Mu *et al.*, 2005; Wang *et al.*, 2007). A strong dose response was observed in green tea consumption and protection from gastric cancer (Mu *et al.*, 2005). Green tea consumption also showed an inverse relationship with breast cancer. Wu *et al.* and Zhang *et al.* conducted case-control studies respectively in America and China; both studies not only showed an inverse relationship with breast cancer risk, but also showed a dose-response with amount of

green tea intake (Wu *et al.*, 2003; Zhang *et al.*, 2007). Zhong *et al.* also demonstrated inverse relationship and positive dose-response of green tea ingestion with lung cancer with non-smoking Chinese women (Zhong *et al.*, 2001). Besides the antioxidant property, green tea can inhibit carcinogenesis by suppressing signal transduction pathways which involve cell proliferation, such as nuclear factor-kappa B (NF-κB) and epidermal growth factor receptor (EGFR)-mediated pathways (Khan & Mukhtar, 2008). In our previous study, human plasma total antioxidant content increased significantly and rapidly after a single dose of green tea: the FRAP value increased about 4% within 40 mins (Benzie *et al.*, 1999). This may indicate some polyphenolic compounds in green tea are absorbed by the human body and may help lower oxidative modification of DNA.

Bilberry

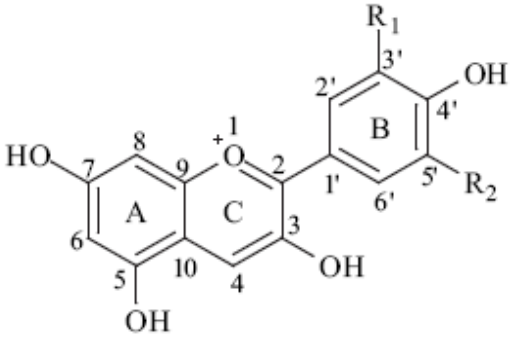
Anthocyanins

Bilberry (*Vaccinium myrtillus L*) is an edible berry which is often confused with blueberry due to their similar appearance. Bilberry is rich in a sub-group of flavonoids, named anthocyanins. Anthocyanins are water soluble pigments and are responsible for the blue, violet and red colour of fruits and vegetables. More than 600 naturally occurred anthocyanins are identified (Prior & Wu, 2006). The basic structure of anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium. Anthocyanins differ from each other in the number and position of hydroxyl and the number and nature of sugars attached (Figure 1.11).The antioxidant activities of anthocyanins and its aglycone form,

named anthocyanidins, seem related to the chemical structure (Kowalczyk *et al.*, 2003). The hydroxyl group at the C ring can chelate metal ions which are responsible for Haber-weiss reaction (Kowalczyk *et al.*, 2003). Furthermore, the hydrogen donating antioxidant ability of anthocyanins is higher when compared to other flavonoids (Zafra-Stone *et al.*, 2007). The absorption of anthocyanins is rapid, and only needs 0.5 hrs - 2 hrs to reach the maximum concentration in plasma (Prior & Wu, 2006). However, the clearance of anthocyanins is also rapid, and the anthocyanins plasma level is almost undetectable after 6 hrs ingestion (Cao *et al.*, 2001). Similar to other phenolic compounds, the bioavailability of anthocyanins is poor (< 0.1 % of the ingested dose) (Prior & Wu, 2006). Even though the bioavailability is poor, a range of pharmacological properties have been reported which include antioxidant (Møller *et al.,* 2004), anti-tumour (Hou, 2003), anti-inflammatory (Dreiseitel *et al.*, 2008), hypoglycaemic (Martineau *et al.*, 2006) and vision improvement effects (Canter & Ernst, 2004).

There are very few studies of bilberry alone, though there are some of the juices of bilberry plus other berries, and there are studies of cranberry and other anthocyanin-rich berries and juices. For example, Pedersen and co-worker report the plasma antioxidant capacity (as the FRAP value) increased significantly after ingestion of 500 ml cranberry juice (Pedersen *et al.*, 2000). Furthermore, anthocyanin concentrates from elderberry showed significant protective effect against H_2O_2 induced DNA damage in human colon cells *in vitro* (Pool-Zobel *et al.*, 1999). Apart from antioxidant properties, anthocyanins also show other pharmacological properties. Hou showed that delphinidin, petunidin and cyanidin

inhibited tumour promoter tetradecanoylphorbol-13-acetate (TPA) transactivation (Hou, 2003). Anthocyanins can also increase insulin secretion (Zhang *et al.*, 2004) and promote vasorelaxation (Nakamura *et al.*, 2002), which may help to prevent microangiopathy development in diabetes patients. Furthermore, blackcurrant anthocyanins can decrease the dark adaptation threshold of vision (Nakaishi *et al.*, 2000). Even though not all studies show beneficial effects, no significant cytotoxic effect was observed (Roy *et al.*, 2002).



Name	R1	R2
Delphinidin	OH	OH
Petunidin	OCH3	H
Cyanidin	OH	H
Pelargonidin	H	H
Peonidin	OCH3	H
Malvidin	OCH3	OCH3

Figure 1.11. Structure of 6 common anthocyanidins.
(Adapted and modified from Figure 1 in Hou DX. Potential mechanisms of cancer chemoprevention by anthocyanins. Curr Mol Med 2003;3(2):149-59.)

To date, there has been no study of effects of bilberry, or any berries, on DNA damage or repair in human trials. However, its high antioxidant content and hypoglycemic effects make it a focus of interest, particularly in relation to a target group that is under increased oxidative stress due to hyperglycaemia, i.e. Type 2 diabetes subjects.

Cordyceps

Cordyceps sinensis (Berk.) Sacc., also knows as Dong Chong Xia Cao in Chinese, is an insect parasitizing fungus. The fungus is especially parasitic of the caterpillar of the Himalayan Bat Moth (*Hepialis armoricanus*) (Holliday & Cleaver, 2008). The whole complex body, including the caterpillar, has been used as a traditional medicine and functional food since ancient times.

Cordyceps contains a wide range of bioactive components including, polysaccharides, nucleosides, amino acids and sterols. In China, Cordyceps is often used as a tonic drug for patients with serious diseases. Recent scientific research shows Cordyceps has antioxidant (Ng & Wang, 2005), antitumour, immunomodulating, antihypertensive (Zhou *et al.*, 2009) and hypoglycaemic effects (Ng & Wang, 2005) and enhances myocardial mitochondrial ATP generation (Siu *et al.*, 2004). Polysaccharides and nucleosides are believed to be the main bioactive components responsible for the pharmacological effects. Due to the increasing reputation of its pharmacological functions, the demand for Cordyceps has markedly increased. However, natural Cordyceps is rare and only grows at high altitudes

(above 3000 m) on the Himalayan Plateau (Zhou *et al.*, 2009). Cordyceps strains have been successfully cultivated worldwide, and Cordyceps mycelium cultures have become the major source for commercial Cordyceps products (Paterson, 2008). Studies show that cultivated Cordyceps has similar pharmacological and chemical compositions as natural Cordyceps (Li *et al.*, 2001).

Polysaccharides

Polysaccharides are one of the major bioactive components of Cordyceps and account for 3-8% of dry weight. Polysaccharides can be extracted from the natural fruit bodies, culture medium and mycelia biomass from mycelium cultures (Zhou *et al.*, 2009). Even though the pharmacological action of polysaccharides remains unclear, a characteristic D-Mannitol, also known as cordycepic acid, is found in Cordyceps (Figure 1.12). Cordycepic acid shows antioxidant power and has been used as a marker for quality control purposes (Li *et al.*, 2006). A water soluble protein containing galactomannan with molecular weight around 23 kDa was extracted from Cordyceps. The galactomannan only contains a small amount of protein and is mainly constructed of D-mannose and D-galactose in a ratio of 3:5. The galactomannan contains (1→6) and (1→2)-linked α-D-mannopyranosyl residues in the main chain (Zhou *et al.*, 2009). Li and co-workers demonstrated that a polysaccharide called CSP-1 possesses a strong protective effect against H_2O_2 induced oxidative damage in rat pheochromocytoma PC12 cells (Li *et al.*, 2003).

Nucleosides

More than 10 nucleosides have been isolated from Cordyceps, including adenine, adenosine, uracil, uridine, deoxyuridine, guanidine, guanosine, thymine, thymidine and inosine (Li *et al.*, 2006). The 3'-deoxyadenosine, also known as cordycepin is a characteristic derivative of adenosine first isolated from *Cordyceps militaris*, which is another closely related species of *Cordyceps sinensis* (Figure 1.10) (Zhou *et al.*, 2009). The presence of cordycepin in *Cordyceps sinensis* is still contradictory. However, Guo *et al.* showed that cordycepin is also present in *Cordyceps sinensis* (Guo *et al.*, 2006). Cordycepin shows anti-tumour activity, inducing apoptotis in cancer cells (Thomadaki *et al.*, 2008) and inhibits UVB-induced NF-κB activation (Lee *et al.*, 2009).

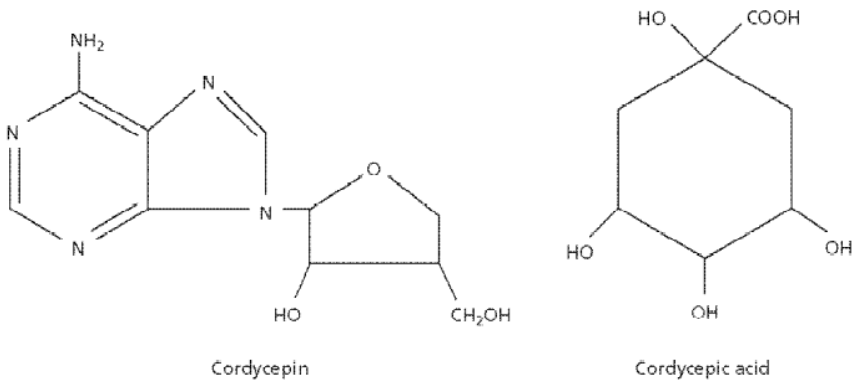


Figure 1.12. Chemical structure of cordycepin and cordycepic acid in *Cordyceps sinensis*.
(Adapted from Figure 1 in Zhou X, Gong Z, Su Y, Lin J, Tang K. Cordyceps fungi: natural products, pharmacological functions and developmental products. J Pharm Pharmacol 2009;61(3):279-91.)

Summary And Focus Of The Study

The association between increased oxidative stress and many age-related diseases, such as cancer, diabetes, and cardiovascular diseases, is established. Mechanisms are unclear, but a key factor is believed to be oxidation-induced modification/damage to biomolecules, such as DNA, protein and lipid. DNA contains the genetic material responsible to maintain normal metabolism. Therefore, maintenance of DNA integrity is important for attenuating risk of age-related diseases and for health promotion. This can be achieved by increasing antioxidant, or other defences, against damage, and/or by promoting DNA repair.

Epidemiological and *in vitro* experimental studies show that some antioxidant-rich foods and herbs have genoprotective or anti-cancer effects. Many traditional herbs and remedies, such as green tea, Lingzhi, bilberry and Cordyceps are suggested to have antioxidant and may have genoprotective effects. However, the scientific finding on selected herbs on genoprotection is lacking. The main focus of this study was to investigate the genoprotective effects of selected herbs either by *in vivo* or *in vitro* studies by using various versions of the comet assay. The aims and objectives of the study will be presented in the next chapter.

Chapter 2

AIMS AND OBJECTIVES OF THE STUDY

The overall aim of this study was to investigate the genoprotective and DNA repair effects of selected 'herbs' or functional foods by using various versions of the comet assay. Within this overall aim, the study had 4 main objectives, each of which employed a different version of the comet assay and which focused on a different herb/functional food. The specific objectives were:

1. To investigate the acute protective and repair effects of *Ganoderma lucidum* (Lingzhi) on lymphocytic DNA by using the 'standard alkaline' comet assay, and the timed repair version of the oxidant challenge comet assay in a human trial.

2. To investigate, in human study, the genoprotective effect of regular intake of *Camellia sinensis* (green tea) on nucleotide (urine 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxodG)) and DNA (lymphocytic 8-oxo-7,8-dihydroguanine (8-oxoGua)) level by using LC-MS/MS and the formamidopyrimidine-DNA glycosylase (Fpg)-assisted comet assay, respectively, and to investigate the relationship between these two biomarkers.

3. To investigate the effect of *Vaccinium myrtillus L* (bilberry) anthocyanins on base excision repair (BER) pathway in type 2 diabetes patients using a lymphocyte extract version of the comet assay.

4. To investigate, using a normal human fibroblast cell line, the potential photoprotective effect of *Cordyceps sinensis* mycelium extract against ultraviolet B (UVB)-induced DNA damage in normal human fibroblasts, using the T4 endonuclease V (T4EV)-assisted comet assay.

Chapter 3

MATERIALS AND METHODS

In this study six versions of the comet assay were used: i) the standard alkaline comet assay; ii), the H_2O_2 oxidant challenge version; iii) the timed repair from challenge version; iv) the Fpg version for oxidation-induced damage; v) the T4EV model for CPDs induced by UVB; vi) the cell extract version with damaged substrate cells, used for hOGG1 repair activity. Some versions were used alone in different experiments, some in combination. The comet assay was run mainly on cryopreserved human lymphocytes harvested from heparinised venous blood. To avoid duplication in content of chapters 4-7, all the comet assay methods are described in detail in this chapter, along with details of lymphocyte harvesting and cryopreservation. With regard to other biomarkers tested, such as urine 8-oxodG, methods are described in detail in the relevant chapter. With regard to different experiments, the statistical analyses are described in the relevant chapters.

Harvesting And Cryopreservation Of Peripheral Lymphocytes

Reagents

Reagents for harvesting of lymphocytes included RPMI 1640 and foetal bovine serum (FBS) purchased from Gibco-Biocult (Grand Island, NY, USA) and Histopaque 1077 and dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich

(St. Louis MO, USA). All reagents were of molecular biology grade or highest purity grade available.

Method

Preparation And Harvesting Of Lymphocytes (i: from whole blood)

One ml heparinised blood was added to 1 ml pre-chilled RPMI 1640 medium and underlaid with 2 ml Histopaque 1077 (15 ml centrifuge tube). The 15 ml centrifuge tube was centrifuged for 30 mins at 1200 rpm at room temperautre without the brake on. The buffy coat, containing the lymphocytes, was retrieved by harvesting the layer just above the Histopaque 1077 layer, and care was taken to avoid taking red blood cells along with the lymphocytes. The harvested lymphocytes layer was transferred to a second 15 ml centrifuge tube. One ml of RPMI was added, and the tube centrifuged for 10 mins at 1200 rpm. After centrifugation as much supernatant as possible was removed. If cells could not be tested in the comet assay immediately, they were cryopreserved following an established protocol (Collins *et al.*, 1997) and as described below.

Preparation And Harvesting Of Lymphocytes (ii: from buffy coat)

This method was used when both plasma and lymphocytes were required from the same blood sample. The blood sample (4 ml heparinised blood in commercial blood tube) was centrifuged for 15 mins at 1500 rpm at 4 ^{o}C. Plasma was removed and replaced with 2 ml RPMI. The buffy coat layer plus the 2ml of RPMI were transferred to a 15 ml centrifuge tube, mixed with an equal volume of

RPMI and underlaid with 4 ml Histopaque 1077. The 15 ml centrifuge tube was centrifuged for 15 mins at 1200 rpm at 4 ^{o}C without the brake on. The lymphocytes was retrieved by harvesting just above the Histopaque 1077 layer. The lymphocytes were transferred to a new 15 ml centrifuge tube and washed twice with 5 ml RPMI .

Cryopreservation And Thawing Of Lymphocytes

The cell pellets were resuspended in freezing medium (90 % FBS with 10 % DMSO) and transferred to a labelled micro-centrifuge tube (~ 500 µl). Micro-centrifuge tubes were placed in a thick-walled styrofoam box in a –80 ^{o}C freezer and stored at this temperature (Collins *et al.,* 2001c). On the day of testing, the cells were thawed quickly by holding a tube in a 37 ^{o}C water bath. One ml RPMI was added and centrifuged as before and the RPMI was discarded. The cell pellet was re-suspended in 20 µl cold PBS. The cell suspension was split, as necessary, into two 10 µl aliquots. One 10 µl aliquot was subjected to oxidant challenge, and the other 10 ul aliquot was subjected to PBS as a control as described below.

The Standard Alkaline Version, The H_2O_2 Challenge Version And The Timed Repair Version Of The Comet Assay

Materials

Reagents and chemicals for the alkaline comet assay included agarose 3:1, purchased from Ameresco (Ohio, USA), hydrochloric acid (HCl), purchased from

Merck, (Darmstadt, Germany), Type VII low gelling point (LMP) agarose, disodium ethylenediaminetetracetic acid dihydrate (Na_2EDTA), sodium chloride (NaCl), sodium hydroxide (NaOH), phosphate buffered saline powder (PBS; pH 7.4), hydrogen peroxide solution, Triton X-100, Tris(hydroxymethyl) aminomethane (Tris) and ethidium bromide, all purchased from Sigma-Aldrich. All reagents were molecular biology grade or highest purity available. All reagents were prepared by using Milli-Q water (Milli-Q® Synthesis A10^{TM}, Millipore, Molsheim, France). This is highly purified water (18.2 megohms)

Method

Slide Preparation

The standard alkaline comet assay was performed following well established protocols (Duthie *et al.*, 1996; Duthie *et al.*, 2002; Wong *et al.*, 2005; Collins, 2009). Clean glass microscope slides (Sail, China) were precoated with agarose by dipping slides into melted 1% (w/v in PBS) standard agarose solution. The excess agarose was drained off and the backs of slides were wiped with a tissue. The slides were air dried and stored at room temperature in a paper box until needed. Eighty-five µl of warm 1% (w/v in PBS) standard agarose in PBS was pipetted onto the top and also the bottom part of the agarose pre-coated side of test slides on day of testing. Cover slips were applied, and slides were then placed in the fridge for at least 5 mins for the agarose to solidify. These slides were then used in all versions of the comet assay.

Embedding Lymphocytes In Low Melting Point (LMP) Agarose

An aliquot of washed lymphocytes (fresh or cryopreserved, typically each aliquot would contain cells from 50 µl of whole blood) was mixed with 85 µl 1 % (w/v in PBS) pre-warmed (37^{o}C) LMP agarose and quickly transferred to one of the pre-coated slides prepared as described above. A cover slip was placed on each cell-containing gel, and the slide was placed in the fridge for at least 5 mins until the agarose solidified. As soon as the gel had solidified, the slides were advanced to the next step in the procedure. In the standard alkaline comet assay the next step was cell lysis.

Lysis

On the day of use, 400 µl Triton X-100 was added to 40 ml of cold lysis solution (0.1 mol/l Na_2EDTA , 2.5 mol/l NaCl and 10 mmol/l Tris, adjusted to pH 10 by adding concentrated NaOH) and this lysis solution plus Triton X-100 was mixed for at least 30 mins at 4 ^{o}C. The solution was added to a clean Coplin jar. After cover slips were removed, slides from the previous step were immersed in the solution at 4^{o}C for 1 hr in order to lyse the cell and nuclear membranes and remove histones from around the DNA.

Alkaline Treatment

Forty ml of cold electrophoresis solution (0.3 mol/l NaOH and 1 mmol/l Na_2EDTA, pH >13) was added to a clean Coplin jar; slides were removed from the lysis solution and immersed in this solution at 4°C for 20 mins x 2 changes for DNA unwinding.

Electrophoresis

Slides were removed from the electrophoresis solution and placed horizontally on the platform in an electrophoresis tank (Thistle Scientific, Glasgow, UK). The tank already contained cold electrophoresis solution. Slides were laid in complete rows (gaps if any were filled with blank slides). Care was taken to ensure the slides were just covered by cold electrophoresis solution. Electrophoresis was run for 30 mins in the cold room at 25 V; current was set to 0.30 A by adjusting the electrophoresis solution level.

Neutralization

After electrophoresis, slides were removed from the tank and immersed in neutralizing buffer (0.4 mol/l Tris, adjusted to pH 7.5 by adding concentrated HCl) at 4°C for 5 mins. The buffer was drained and the neutralizing step was performed two more times with fresh solution. The slides were removed from neutralizing buffer and air dried. All slides were stored in a slides box until scoring (for summary of protocol, see Figure 3.1).

Staining And Scoring

Slides were stained just before scoring with 40 µl of 2 µg/ml ethidium bromide in water and examined with fluorescence microscopy (Nikon Eclipse E600, Nikon, Tokyo, Japan) with excitation and emission filters of 510-560 nm and 575 nm respectively. Computerized image analysis (Kinetic Komet 5.5, UK) was used to score damage immediately after staining. The % tail DNA content was measured as the index of DNA damage, and 50 cells per slide for each treatment were measured; each treatment was performed in duplicate (i.e, DNA damage in 100 cells per treatment was scored).

H_2O_2 Treatment For Induction Of DNA Damage

In the 'H_2O_2 challenge' version, cells were exposed to a standard oxidant challenge immediately before being embedded in agarose. This version of the comet assay is used to assess resistance of whole cells to *in vitro* oxidant challenge under controlled conditions (Wong *et al.,* 2005). In this study, 1 ml freshly prepared 30 µM hydrogen peroxide in PBS was added to one 10 µL aliquot of cell suspension (in an Eppendorf tube) in order to induce an oxidant challenge. One ml of cold PBS was added to another 10 µl aliquot of the same cell suspension as a control. Both tubes were kept on ice for 5 mins. After that, the tubes were centrifuged as described above. Supernatant was removed, and challenged cells and control cells were washed with 1 ml cold PBS, then centrifuged as before. After centrifugation, the supernatant was removed and the cell pellets were re-suspended in cold PBS

(typically 30 µl). A 10 µl aliquot of each cell suspension was immediately embedded in LMP agarose. The lysis, unwinding, electrophoresis and scoring steps as described above were performed immediately after embedding, with control and challenged cells run in parallel. In some experiments, aliquots of challenged cells were allowed to repair under controlled conditions, as described in the next section.

DNA Repair In Challenged Cells (60 and 180 mins repair periods) (Collins, 2004)

The oxidation-induced damage is normally repaired quite rapidly. To assess the rate and amount of repair of BER (which is the pathway mainly involved in repair of oxidation induced damage), cells exposed to H_2O_2 were incubated at 37^oC in complete medium and tested using the standard alkaline comet assay after timed periods (in this study, 60 and 180 mins were used). In this repair version of the comet assay, 1 ml pre-warmed RPMI 1640 medium containing 10 % FBS was added to each 10 µl aliquot (i.e. total four aliquots, two of H_2O_2 treated cells and two of control cells). One aliquot each of H_2O_2 treated and control cells were incubated at 37 ^oC with 5 % carbon dioxide for 60 mins. The other set of H_2O_2 treated and control cells was incubated for 180 mins. After this repair incubation, cells were centrifuged, washed with 1 ml cold PBS and centrifuged as before. After centrifugation, as much of the supernatant as possible was removed. The pelleted cells were mixed with 1 % (w/v) pre-warmed LMP agarose in PBS and embedded on microscope slides, and the alkaline comet assay run as described above. The protocol for these versions (a) standard alkaline comet assay b) H_2O_2 challenge and c) timed repair versions is summarized in Figure 3.1

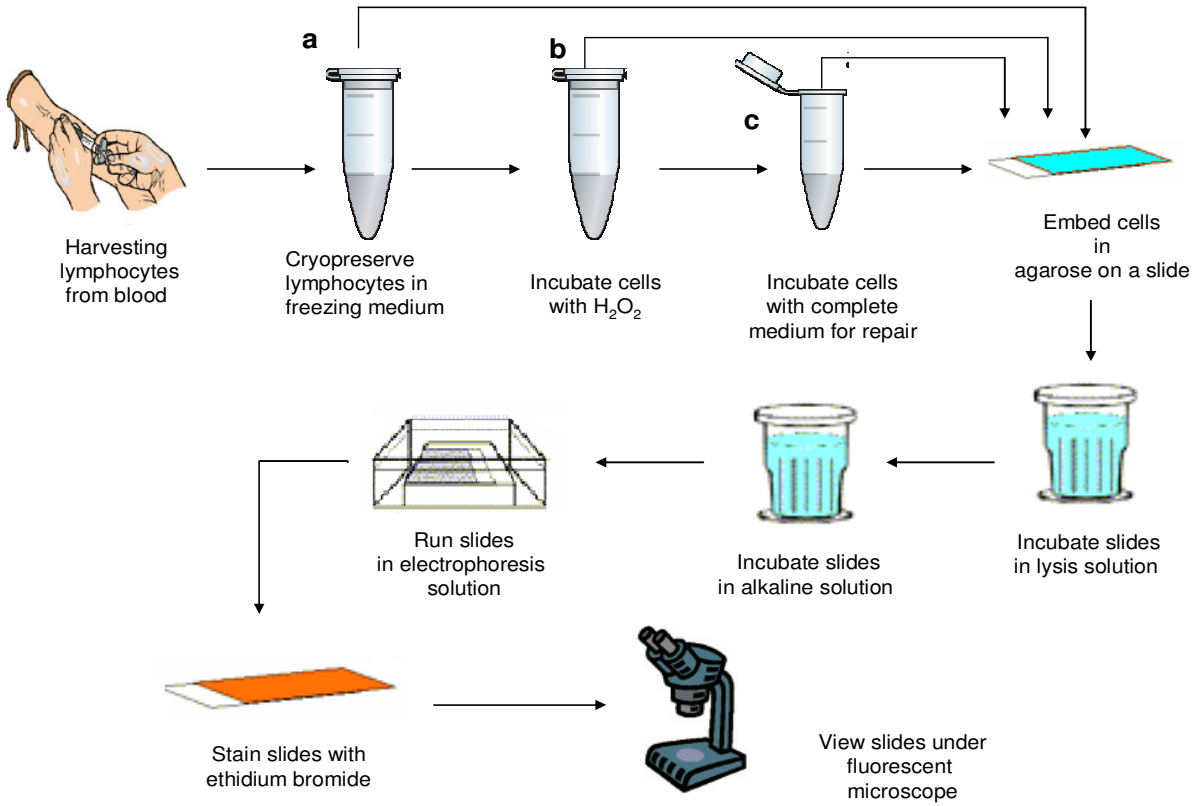


Figure 3.1. The summary protocol of the a) standard alkaline comet assay with b) H_2O_2 challenge and c) time repair steps.

Formamidopyrimidine-DNA Glycosylase (Fpg)-Assisted Comet Assay For Oxidation-Induced Damage To DNA

Materials

The chemicals for the Fpg-assisted comet assay are the same as for the standard alkaline comet assay with some additions. Additional chemicals used in this part of the study included HEPES, potassium chloride (KCl), potassium hydroxide (KOH) and bovine serum albumin (BSA), all purchased from Sigma-Aldrich. The Fpg enzyme was a kind gift from Prof Andrew Collins, Department of Nutrition, Faculty of Medicine, University of Oslo, Norway. As advised by Prof Collins, the enzyme solution supplied was kept at -80^{o}C and when needed was thawed and diluted 1/3000 in enzyme buffer for use.

Method

The enzyme Fpg creates strand breaks at sites of 8-oxoGua (the major substrate), 4,6-diamino-5-formamidopyrimidine (FapyAde) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua). While not completely specific for 8-oxoGua, the use of the enzyme in the Fpg-assisted comet assay markedly increases the sensitivity and specificity of the comet assay for oxidation-induced DNA damage (Collins, 2009). In the Fpg-assisted comet assay, the enzyme-treatment step is introduced immediately after the lysis step, as follows.

Following the lysis step, slides were transferred into a staining jar containing 40 ml Fpg enzyme reaction buffer (40 mM HEPES, 0.1 M KCl , 0.5 mM Na_2EDTA, 10 mM Tris, 0.2 mg/ml BSA , adjusted to pH 8.0 by adding KOH) and left for at least 5 mins. Slides were removed and drained, and 55 µl of working Fpg enzyme solution (stock supplied diluted 1/3000 in enzyme buffer as described above) or enzyme buffer alone (as control) was added to each gel, gels were covered with Parafilm™. Slides were placed in a box containing moist tissue and incubated at 37 °C for 30 mins. After incubation, slides were immediately processed through alkaline treatment, electrophoresis and neutralization steps as for the standard version of the alkaline comet assay described above.

It is important to note that the cells tested in the Fpg-assisted comet assay are not challenged with H_2O_2.

T4 Endonuclease V (T4EV)-Assisted Comet Assay For UVB-Induced Damage To DNA

Materials

Chemicals for the T4EV-assisted comet assay are same as for the standard alkaline comet assay. Additional chemicals used in this part of the study included T4 endonuclease V (T4EV) enzyme purchased from Epicentre Technologies (Madison, WI, USA). Highest purity available reagents were purchased. Eagle's Minimum Essential Medium (EMEM), FBS, sodium pyruvate, and trypsin-EDTA

(0.25 % trysin, 1 mM Na_4EDTA) were from Gibco-Biocult, PBS; pH 7.4 and trypan blue dye were from Sigma-Aldrich.

Method

The enzyme T4EV creates strand breaks at a particular type of lesion, the CPDs. These lesions are a sign of UVB induced damage (Marrot & Meunier, 2008). Specificity is not 100%, however, the use of T4EV enables the comet assay to be used to 'probe' for UVB-induced damage and to investigate the effect of potentially protective agents. In theory, various cells can be used in this model, including lymphocytes. However, as the main target tissue of UVB damage is skin, a relevant cell type is a normal (untransformed) skin cell, or fibroblast. In this study, the cell type that was used was a human normal foreskin fibroblast cell line called BJ. These cells were purchased from the American Type Culture Collection (Manassasa, VA, USA). The cells were kept in liquid nitrogen until thawed and cultured as described below.

BJ Cells Preparation

The BJ cells were maintained in EMEM, supplemented with 10 % FBS and 1 mmol/l sodium pyruvate. Cell cultures were established in 75 cm^2 flasks and kept in a humidified atmosphere with 5 % CO_2 at 37 ^oC. Medium was replaced every 3 days. All cells tested had a population doubling number of 35-55. Cells were removed from the dishes by gentle trypsinization as follows. Liquid culture medium was removed, the cell layer was rinsed with PBS to remove trypsin

inhibitors in remaining medium, and 500 ml trypsin-EDTA solution was added to the culture dish. The dish was placed in an incubator at 37 ^{o}C for 5 mins, then 3 ml of complete medium was added to the dish and cells were aspirated by gentle pipetting. Cell suspension was transferred to a 15 ml centrifuge tube and spun at 1000 rpm for 5 mins at room temperature. The liquid medium was removed and the cell pellet was rinsed twice with PBS. Cells were then resuspended in complete culture medium. Cells (4.5 x 10^5 BJ fibroblasts) were seeded onto 60 mm culture dishes. After overnight incubation in complete medium, culture medium was removed and cells were rinsed with PBS and incubated with defined concentration of test agent [<500EPSCM or HWECMyc (50, 100, 200 µg/ml), see Chapter 7] in complete medium for 30 mins and 24 hrs. These were supplied as freeze dried powders by Dr. JY Wu, Department of Applied Biology & Chemical Technology, The Hong Kong Polytechnic University. One was a hot water extract of dried mycelia biomass, referred to as 'HWECMyc'. The other preparation was a crude extract prepared as a 95 % v/v ethanol precipitate of the liquid medium around the cultured mycelia. Molecular weight cut-off membranes were used to collect extract with exopolysaccharides of molecular weight < 500kDa and this is referred to as '<500EPSCM'.Cells were incubated with complete medium alone as control. After incubation, medium was removed and cells were rinsed twice with PBS. Cells from each treatment tube were counted by haemocytometer and cell viability was determined by the Trypan blue exclusion test using a Beckman Coulter Vi-Cell XR analyzer (Miami, FL, USA). Remaining cells in each treatment tube were resuspended in 1 % (w/v) low melting point agarose in PBS to a final concentration

of ~600 cells in 100 µl cell suspension, and 85 µl of this agarose cell suspension was applied to a microscope slide as described in the standard alkaline version of the comet assay. Cells were then subjected to UVB irradiation and as described in Chapter 7, before the T4EV-assisted comet assay was run as described below, based on the procedure of Sauvaigo *et al* (1998) with some modifications.

Running The T4EV-Assisted Comet Assay

Cells embedded in LMP agarose, and for measurement of UVB-induced damage were lysed as described above in the standard alkaline comet assay. After lysis, the slides were washed 3 x 5 mins with cold (4 ^{o}C) T4EV enzyme reaction buffer (50 mmol/l Tris-HCl and 5 mmo/l Na_2EDTA, adjust pH to 7.5 with HCl). Fifty µl of 0.01 unit of T4EV in this buffer (or buffer alone as a control) was then added to each gel, and the gel was covered with a piece Parafilm™. Slides were placed on moist tissue in a box and incubated in this humidified atmosphere at 37 °C for 15 mins. After incubation, the Parafilm™ cover was removed. Slides were immediately processed through the alkaline treatment, electrophoresis and neutralization steps as in the standard version of the alkaline comet assay. An overview of the T4EV protocol is shown in Figure 7.2 (Cordyceps chapter).

The Cell Extract Version Of The Comet Assay, With Damaged Substrate Cells, Used For hOGGI Repair Activity

Materials

The chemicals for this version of the comet assay are the same as for the standard alkaline comet assay with some additions. Additional chemicals used in this part of the study included HEPES, KCl, KOH and BSA. All were purchased from Sigma-Aldrich; dithiothreitol was purchased from Fluka (Seelze, Germany); glycerol was purchased from Riedel-de-Haen (Seelze, Germany). Highest purity available for all reagents was purchased. RPMI 1640, FBS, and trypsin-EDTA (0.25% trysin, 1mM Na_4EDTA) were purchased from Gibco-Biocult. PBS; pH 7.4 was purchased from Sigma-Aldrich. The photosensitizer Ro 19-8022 was a kind gift from F. Hoffmann-La Roche. The HeLa cells were a kind gift from Dr. Polly Hang Mei Leung, Department of Health Technology & Informatics, The Hong Kong Polytechnic University.

Method

In this version of the comet assay, a cell extract is prepared. This cell extract contains the DNA repair enzymes. Of interest here is hOGG1, which is involved in the first step of BER. hOGG1 is the human equivalent of Fpg, and creates a strand break at a site of DNA damage, mainly oxidized guanine but also AP sites (Tchou *et al.*, 1994; Collins *et al.*, 2001b). The concept used in this version of the comet assay

is that, when the cell extract is added to lysed, damaged 'substrate cells', the hOGG1 in the cell extract recognizes these damaged sites and create strand breaks in preparation for repair. The greater the hOGG1 activity, the more strand breaks will be induced per unit time in damaged cells. Therefore, the hOGG1 reveals pre-existing damage. When the cell extract is used on cells that contain a 'standard' amount of damage, the amount of damage revealed is a function of the hOGG1 activity. HeLa cells are robust and offer a cell model that can be damaged and used to assess hOGG1 activity in cell extracts, these cell extracts being made from cells of interest, such as lymphocytes that have been 'pre-treated' (in vitro or by supplementation) with an agent that might increase hOGG1 activity. As hOGG1 recognizes predominantly lesions that are oxidation-induced, the HeLa substrate cells are subject to an oxidant challenge prior to treatment with the cell extract. A model of damage induction for use in this version of the comet assay is photosensitization, in which a photosensitizer (Ro 19-8022) is used. Preparations of the photosensitizer, the cell extract and damaged substrate cells, and the comet assay protocol for this version of the assay are described below.

Preparation Of Photosensitizer Ro 19-8022 (Collins et al., 2001b)

Ro19-8022 was dissolved in 70 % ethanol at 1 mmol/l concentration and stored in an aluminum foil wrapped micro-centrifuge tubes at -20 ºC. Excessive light was avoided during preparation. An aliquot of stock Ro 19-8022 was diluted with cold PBS to 1 μmol/l immediately before use.

Substrate Cells Preparation

HeLa cells were maintained in RPMI 1640, supplemented with 10 % FBS. Cell cultures were established in 100 mm culture dishes and kept in a humidified atmosphere with 5 % CO_2 at 37 ^{o}C. Medium was replaced every 3 days. Near-confluent cultures of cells were seeded onto 60 mm culture dishes with 3 x 10^5 cells. After overnight incubation, culture medium was removed and cells were rinsed with PBS and incubated with 5 ml of 1 μmol/l Ro 19-8022 in cold PBS and irradiated on ice for 5 mins at 33 cm from a 250 W tungsten halogen lamp. After irradiation, Ro 19-8022 was removed and cells were washed twice with cold PBS, light was avoided during substrate cells preparation except for the halogen lamp treatment step. Cells were removed from the dishes after gentle trypsinization performed by adding 500 μl of trypsin-EDTA solution. The dish was placed in an incubator at 37 ^{o}C for 5 mins, then 3 ml of complete medium was added to the dish and cells were aspirated by gentle pipetting. Cell suspension was transferred to a 15 ml centrifuge tube and spun at 1000 rpm for 5 mins at 4 ^{o}C. The liquid medium was removed and the cell pellet was rinsed twice with PBS. Cells density was counted by haemocytometer. HeLa cells were suspended in freezing medium (90 % FBS with 10 % DMSO) at a density of 3 x 10^6 cells/ml. Cell suspension was split into 0.4 ml aliquots (enough to make 28 slides) in microcentrifuge tubes and placed in thick-walled styrofoam box in a –80 ^{o}C freezer until needed for the assay. Aliquots were thawed as needed on the day of testing. In order to ensure the similarly of substrate cells, same batch of substrate cells was used throughout this part of study.

Preparation Of Cell Extract

Lymphocytes separation from venous blood used Histopaque 1077 was as described earlier. The cell pellet in a centrifuge tube was mixed with 5 ml of cold PBS and cell density was determined by haemocytometer. The centrifuge tube was spun at 1500 rpm for 15 mins at 4 ^{o}C and the PBS removed. The cell pellet was resuspended in cold PBS at 3.5 x 10^{6} per ml and transferred to 1.5 ml micro-centrifuge tube. The micro-centrifuge tube was spun at top speed in a micro-centrifuge for 5 mins at 4 ^{o}C. The supernatant was removed as much as possible so that the cell pellet is almost dry. This is crucial because leftover PBS will cause a dilution of hOGG1 activity in the cell extract when this is made later. The cell pellet was flash frozen in liquid nitrogen and stored at -80°C, under which conditions hOGG1 activity is stable for at least 1 month (Collins *et al.*, 2001b).

As needed, aliquots of the lymphocytes from the previous step were thawed on the day of testing, and 65 µl of extraction buffer (45 mM HEPES, 0.4 M KCl , 1 mM Na_2EDTA, 0.1 mM dithiothreitol, 10% glycerol, adjusted to pH 7.8 by adding KOH) containing 0.25 % Triton X-100 was added just before use to complete the cell lysis. After vortex-mixing at high speed for 5 sec, the micro-centrifuge tube was put on ice for 10 mins. The lysates were spun at high speed in a micro-centrifuge for 5 mins at 4 ^{o}C. Fifty-five µl of supernatant was removed and mixed with 220 µl of cold enzyme buffer (40 mM HEPES, 0.1 M KCl , 0.5 mM Na_2EDTA , 0.2 mg/ml BSA , adjusted to pH 8.0 by adding KOH).

Reaction Of Cell Extract On Damaged Substrate Cells

An aliquot of substrate cells was thawed quickly and washed twice with PBS. The cell pellet was suspended in 100 µl of PBS and mixed with 4 ml of 1% (w/v in PBS) LMP agarose at 37°C. Eighty-five µl of this agarose cell suspension was applied to a microscope slide as described in the standard version of the alkaline comet assay and cells were lysed, also as described before. After lysis, slides were washed 3 times (5 mins each) with cold enzyme buffer in a staining jar. Fifty-five µl of cell extract was added to each gel and the gel was covered with a piece of Parafilm™. Slides were placed in a box with moist tissue and incubated at 37 °C for 15 mins. After incubation, slides were immediately processed through the alkaline treatment, electrophoresis and neutralization steps as for the standard version of the alkaline comet assay. For summary of protocol, see Figure 3.2.

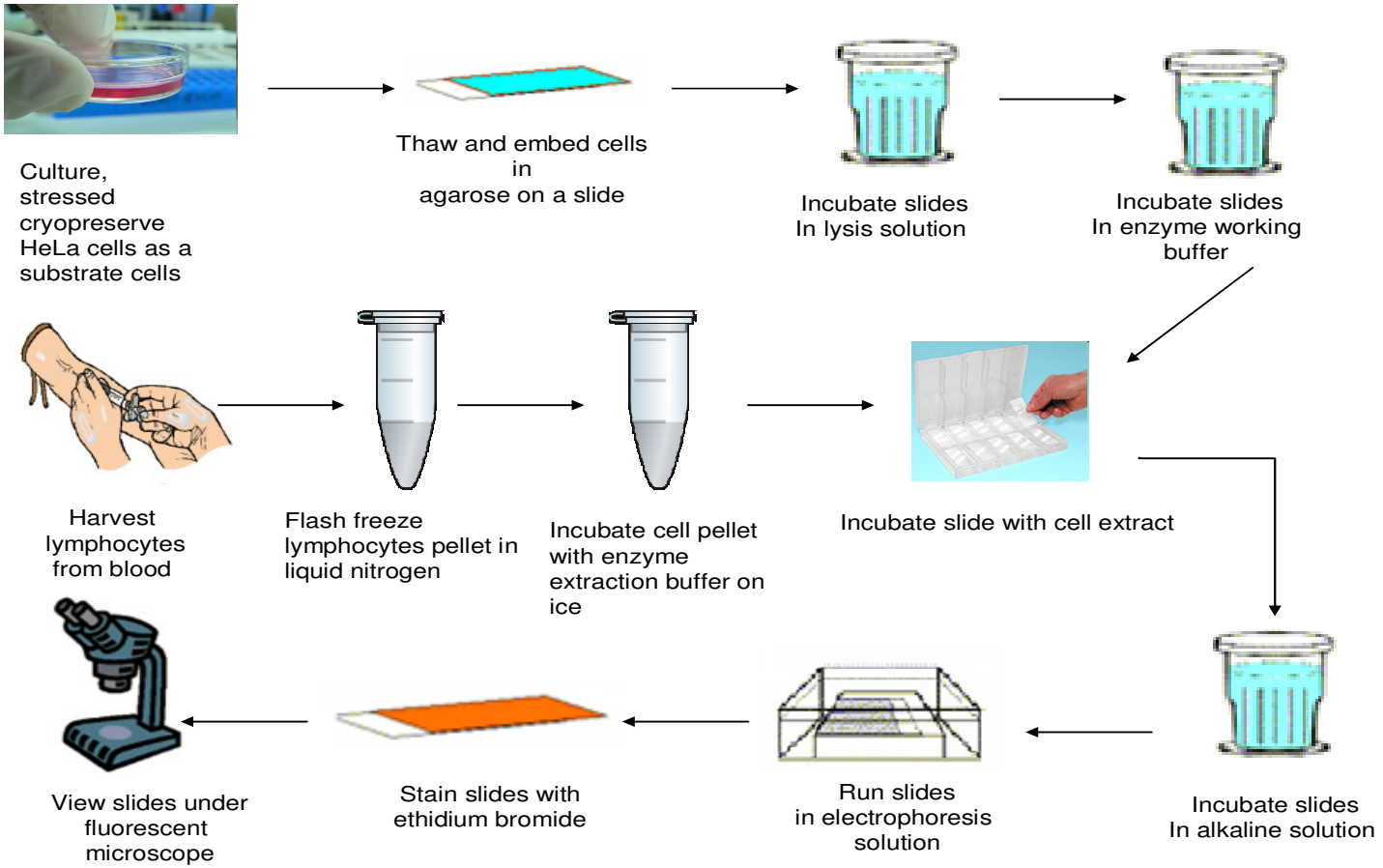


Figure 3.2. Summary of protocol for the cell extract comet assay for DNA repair (hOGG1) activity

Chapter 4

DNA REPAIR EFFECTS AND ANTIOXIDANT POWER OF *GANODERMA LUCIDUM* (LINGZHI) IN HUMAN ACUTE POST-INGESTION STUDY

Introduction:

Due to widely accepted health promoting effects of plant-based diets and nutraceutical products, their global consumption has increased rapidly worldwide during past decades (Upton, 2000; Mahady, 2001). Various phytochemicals and vitamins in plants are reported to have high antioxidant power, which may help to prevent oxidation-induced damage of vital biological molecules, and delay onset of age related diseases (Leung *et al.*, 2006; Møller & Loft, 2006; Szeto & Benzie, 2006; Benzie & Wachtel-Galor, 2009). Different approaches are used to investigate the beneficial effects of plant-based diets, including *in vitro* and *in vivo* supplementation studies with the whole food or extracts.

Mushrooms have been used as an edible and medical resource for thousands years. *Ganoderma lucidum* (Curtis: Fr) P. Karst, also known as 'Lingzhi' in China, is a woody mushroom which is widely used in Asian countries for health promotion (Wachtel-Galor *et al.*, 2004a; Paterson, 2006; Zhou *et al.*, 2007). In China, Lingzhi has been used as herbal medicine since ancient times and has a high reputation as a promoter of health. The usage of Lingzhi is well documented in the Chinese

literature “Compendium of Materia” (Ben Cao Gang Mu) in the 16th century (Lin, 2005). In ancient times, Linzghi was used to strengthen body resistance and promote longevity (Zhou *et al.*, 2007). Nowadays, scientific research suggests Linzghi has antioxidant, antitumour, antiviral, antibacterial, immunomodulatory hypolipidaemic and hypoglycaemic effects (Sliva, 2004; Zhou *et al.*, 2007; Chan *et al.*, 2008). Triterpenes and polysaccharides are believed to be the main components having therapeutic effects (Wachtel-Galor *et al.*, 2004a). In our previous work, we found that plasma and urine antioxidant level (FRAP value) increased significantly after Lingzhi extract ingestion, which indicates that antioxidant components in Lingzhi are absorbed (Wachtel-Galor *et al.*, 2004b). No significant change in the ability of lymphocytic DNA to resist oxidative challenge induced by H_2O_2 was seen in a follow up Lingzhi supplementation study (Wachtel-Galor *et al.*, 2004c). However, the effects of Lingzhi on DNA repair capacity have not been investigated to date. This was the focus of this part of the study.

The outcome of oxidatively modified DNA is dependent on the type and site of the modification (Evans *et al.*, 2004). The potential outcomes include mutation, apoptosis and changes in cellular functions. Most oxidation-induced changes to DNA are likely to be in ‘non-key’ genes, and so are unlikely to disturb cellular function. However, pathological changes ultimately emerge if oxidatively modified DNA is allowed to accumulate (Evan & Vousden, 2001). Therefore, the human body is well equipped with various mechanisms to maintain DNA integrity (Christmann *et al.*, 2003; Madhusudan & Middleton, 2005). Antioxidant molecules

and enzymatic antioxidants can be considered as first line defence against oxidatively modified base formation. DNA repair can be considered as second line defence to eliminate modified DNA bases. Most studies of foods and herbs, including those on Lingzhi, have focused on the ability to protect against DNA damage, however, results on Lingzhi are contradictory (Kim *et al.*, 1999; Chiu *et al.*, 2000; Lee *et al.*, 2001; Lai *et al.*, 2006).

In *in vitro* study, Lai *et al.* showed that a *Ganoderma* extract decreased human serum albumin (5 mg/ml) induced 8-oxoguanine in human proximal tubular epithelial cells, measured by the OxyDNA Assay. The protective effect of *Ganoderma* extract was in a dose response manner in the range of 0-64 µg/ml (Lai *et al.*, 2006). Also in *in vitro* study, Lee *et al.* showed the amino-polysaccharide fraction (G009) of Lingzhi decreased H_2O_2 (generated by UV-induced photolysis) induced strand breakage in bacteriophage ϕX174 supercoiled DNA. The protective effects seemed to be related to the scavenging ability of G009, since G009 showed dose response in inactivation of $O_2^{\bullet -}$ and H_2O_2 (Lee *et al.*, 2001). Kim *et al.* showed Lingzhi polysaccharides (6-180 µg/ml) decreased benzo[α]pyrene (1µg/ml) induced 8-oxodG formation in mouse normal liver NCTC clone-1469 cells *in vitro*, however, the effect was not statistically significant (Kim *et al.*, 1999). In an animal study, Chiu *et al.* showed that after 2 weeks' *Ganoderma* extract (~1g/kg body weight per day) consumption in mice, no significant DNA protective effect against ethyl methanesulfonate (intraperitoneally injected; 300 mg/kg body weight) was seen in mouse lymphocytes, using the comet assay (Chiu *et al.*, 2000). To the best of our

knowledge, there is only one published study (Pero *et al.*, 2005) that has claimed that a mushroom formulation, which included Lingzhi, increased lymphocytic DNA repair after four weeks' supplementation. However, the actual dosage of Lingzhi used was not clearly stated and the conclusion was drawn from a single subject only. Therefore, results from that study were not convincing (Pero *et al.*, 2005).

In summary, studies of Lingzhi on genoprotection are inconclusive, human studies are few, and these and *in vitro* and animal studies have focused on protection against oxidation induced DNA damage only. No previous study has investigated the effect of Lingzhi on DNA repair. Therefore, the main aim of this part of the study was to investigate the acute effect of Lingzhi ingestion on DNA repair in a controlled human study by monitoring the decrease in lymphocyte DNA damage, using the timed repair version of the comet assay after *in vitro* standard oxidation challenge. Other aims were to investigate Lingzhi-associated changes in background DNA damage (using the standard alkaline version of the comet assay), resistance against H_2O_2 induced DNA damage, and plasma 'total' antioxidant capacity.

Materials And Methods

Methods used to investigate DNA repair effects and the ability of DNA to resist oxidative challenge were i) the standard alkaline version comet assay ii), the H_2O_2 oxidant challenge version of the comet assay iii) the timed repair from challenge version of the comet assay. These methods were described in detail in Chapter 3. To recap briefly, the standard alkaline version of the comet assay was used to assess background DNA level of single strand breaks. In the H_2O_2 challenge version of the comet assay, lymphocytes were challenged with 30 μM H_2O_2 immediately before being embedded in agarose, and the total damage (pre-existing plus challenge-induced single strand breaks) was measured by the alkaline comet assay. This version of the comet assay can be interpreted as providing an indication of the antioxidant defence against oxidative challenge. In the timed repair from challenge version, lymphocytes were challenged with 30 μM H_2O_2, washed and incubated in complete medium to repair. The standard alkaline comet assay was then run at certain time points to measure repair of damage. The % DNA remaining in the comet tail after incubation in complete medium was calculated by the following equation:

$$\frac{\%\ \text{DNA in tail after incubation in complete medium}}{\%\ \text{DNA in tail immediately after challenge}} \times 100\ \%$$

In this part of the study, peripheral lymphocytes were harvested from venous blood of healthy young adults before and after supplementation with Lingzhi extract

(supplementation details are given below). The lymphocyte harvesting procedure was described in detail in Chapter 3. The plasma total antioxidant capacity was measured by another member of our research group, and data were retrieved by this investigator. The FRAP assay (Benzie & Strain, 1999) was used to measure this. In this assay, the ability of the sample to reduce Fe^{3+} to Fe^{2+} is measured. The complex ferric-tripyridyltriazine (Fe^{3+}/TPTZ) is in excess, when the complex is reduced by the antioxidant at low pH (pH 3.6), an intensive blue colour develops and measurement of the absorbance can be made at 593 nm. This assay is measuring the electron donating or reducing power of antioxidants in the test sample.

Supplementation Scheme For Acute Effects Of Lingzhi

This was a single-blinded, cross-over intervention trial with 6 apparently healthy Chinese adults (3 males, 3 females), aged between 22 to 35 years. Exclusion criteria included hypertension, history of chronic disease, and taking any regular medication. Written informed consent was obtained from each subject before the study began. Ethical approval for this study was granted by the Ethics Human Subcommittee of The Hong Kong Polytechnic University, and all procedures involving human subjects complied with the Declaration of Helsinki, as revised in 2000 (Appendix 1).

Subjects were allocated on a non-selective basis, to receive a single dose of (i) 3.3g (equivalent to 8.5 capsules) powdered Lingzhi extract with 200 ml warm water or (ii) 200 ml warm water as a control. The Lingzhi dosage used was based on our previous study (Wachtel-Galor *et al.*, 2004b). After 2 weeks' washout period,

the procedure was repeated with subjects crossed-over onto the other treatment. The Lingzhi extract was supplied together with a Certificate of Analysis by Vitagreen Co Ltd Hong Kong. In this study, a 10 ml fasting heparinised venous blood sample from the antecubital vein was collected from each subject, before Lingzhi or control was ingested. Additional venous blood samples were collected into commercial heparinised blood collection tubes at 90 and 180 mins post-ingestion. Subjects remained fasting, except for sips of water, for the entire 180 mins period of sample collection. Blood samples were stored at $4^{o}C$ in the dark until separation, which was within 3 hrs of collection. Plasma was used immediately for measurement of total antioxidant power. Lymphocytes were harvested immediately for the comet assay (fasting and 180 min post ingestion samples). The study protocol is outlined in Figure 4.1.

Statistical Analysis

The Friedman comparison test was used to investigate i) timed post-ingestion changes in plasma antioxidant power in relation to baseline levels and ii) timed related change in %DNA content in tail with baseline DNA levels after H_2O_2 challenge. Wilcoxon matched pairs test was used to compare responses of Lingzhi and water control and to investigate changes in DNA repair effect (% DNA remaining in tail) pre- and post- Lingzhi ingestion. $P<0.05$ was regarded as statistically significant. Prism 4.0 (Graphpad Software, San Diego, CA, USA) was used. The key variable in this study was the %DNA in comet tail after repair in complete medium following challenge with H_2O_2. Published data are not useful for

this due to different concentrations of H_2O_2 used to induce oxidative stress. We therefore performed a preliminary power calculation in relation to delta in SD units, and found that 6 pairs (the number selected for the study) could detect a true difference in the mean 1.43 SD at 80% power and 5% significance level.

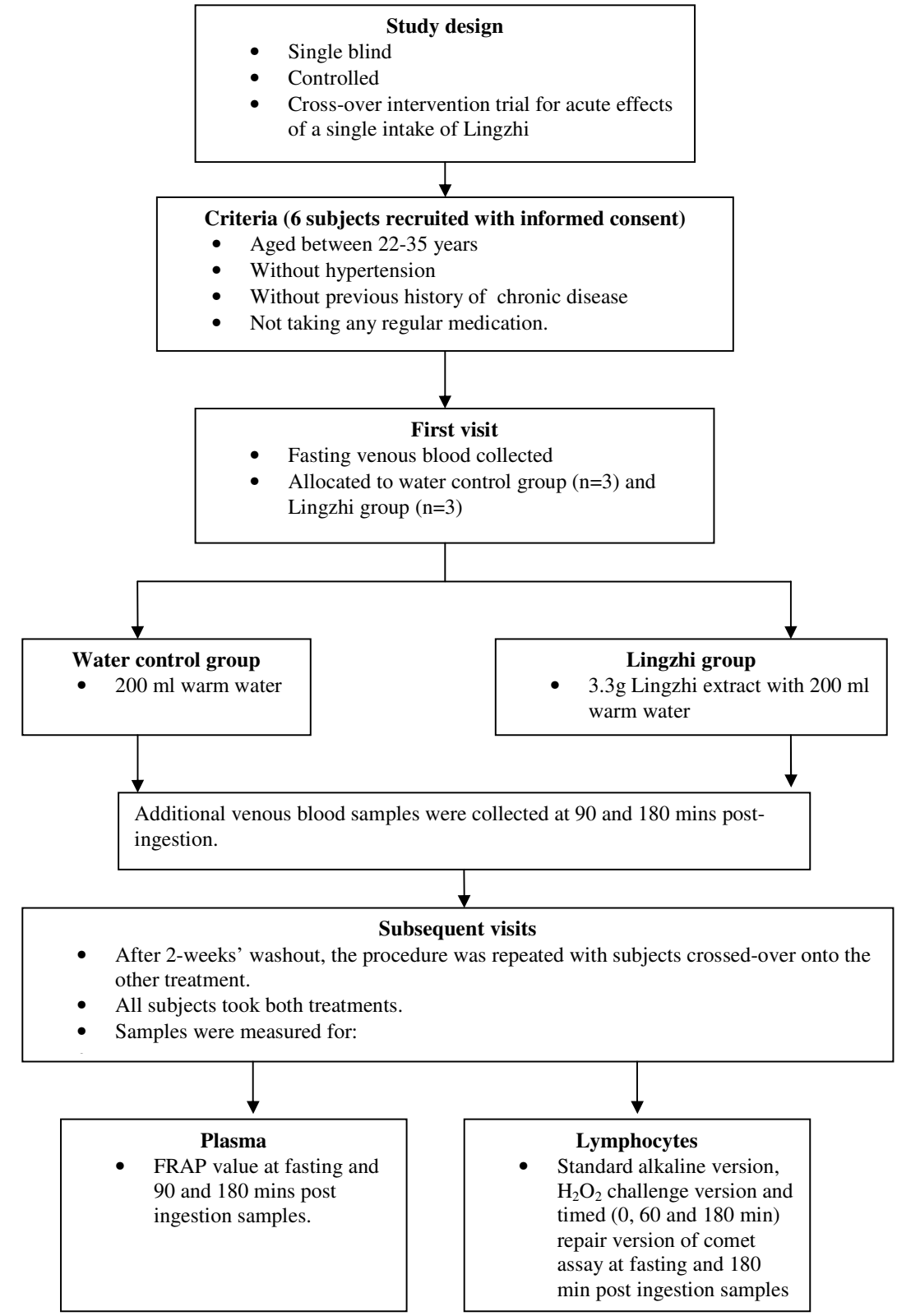


Figure 4.1. Outline of study protocol.

Results

In this acute ingestion bioavailability study, the plasma FRAP value increased, with a peak at 90 mins post-Lingzhi ingestion (Mean +/- SD; 14.08 +/- 18.5), but variation was wide, and this increase was not found to be statistically significant (Figure 4.2). The plasma FRAP value (μmol/l) decreased between 90-180 mins post-Lingzhi ingestion (Figure 4.2). The individual results for plasma FRAP values in the 6 subjects after ingestion of warm water and Lingzhi are shown in Figure 4.3, 4.4 and Table 4.1 and 4.2. It can be seen that the individual variation in plasma FRAP value was quite high.

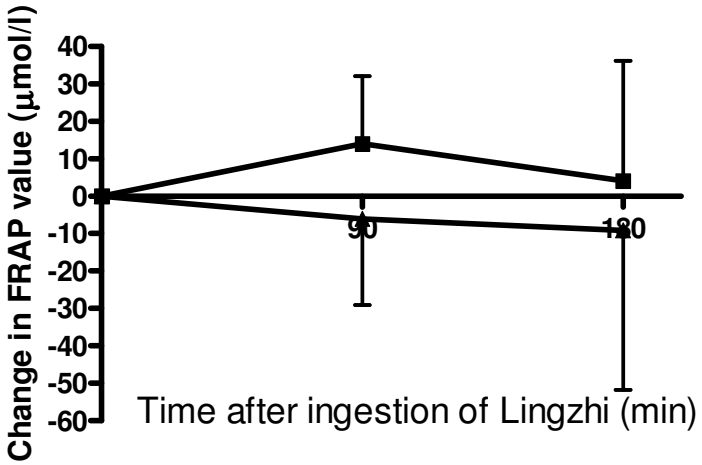


Figure 4.2. The acute changes in plasma antioxidant capacity (n = 6; Mean+/- SD), as FRAP value after ingestion of Lingzhi or water. The square represents Lingzhi treatment and triangle represents water treatment. No significant differences were seen within or across treatments.

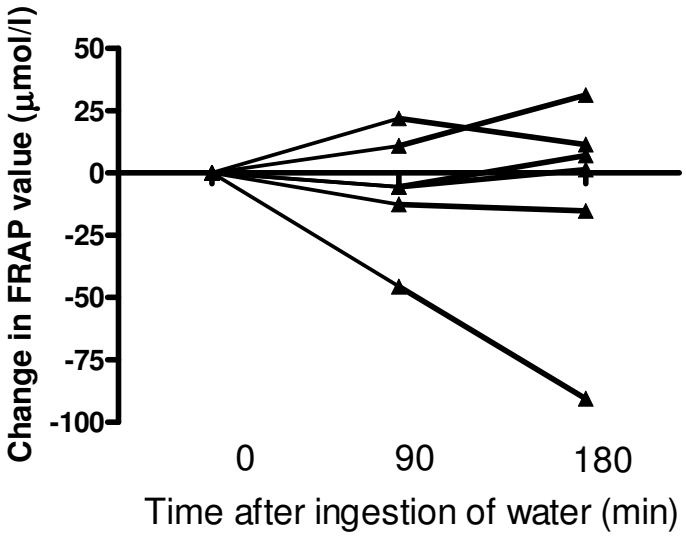


Figure 4.3. Individual results showing changes from baseline in the plasma FRAP value after ingestion of water.

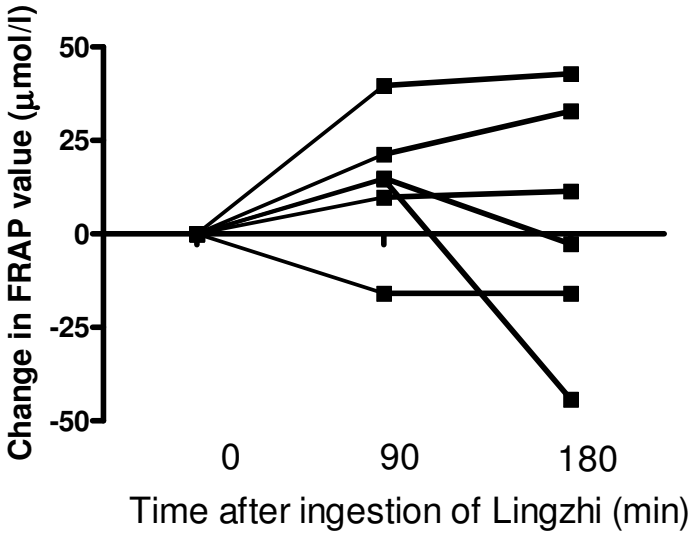


Figure 4.4. Individual results showing changes in the FRAP value after ingestion of Lingzhi.

Table 4.1. Plasma FRAP values at baseline (fasting) and after ingestion of water of individual subjects.

Subject	FRAP value (μmol/l) of plasma at different time point after ingestion of water. Mean of duplicate measurements		
	0 mins (baseline)	90 mins	180 mins
A	1151	1146	1152
B	1243	1197	1152
C	1069	1091	1080
D	833	828	840
E	1062	1050	1047
F	894	904	923
Mean (SD)	1042 (154)	1036 (143)	1032 (127)

Table 4.2. Plasma FRAP values at baseline (fasting) and after ingestion of Lingzhi individual subjects.

Subject	FRAP value (μmol/l) of plasma at different time point after ingestion of Lingzhi. Mean of duplicate measurements		
	0 mins (baseline)	90 mins	180 mins
A	573	594	606
B	1015	1030	1012
C	1318	1332	1274
D	1161	1145	1145
E	1005	1015	1017
F	706	746	749
Mean (SD)	963 (279)	977 (268)	967 (248)

With regard to the background DNA damage level (as strand breaks), the %DNA in tail was similar in fasting blood samples before both Lingzhi and water treatments, and ranged between 2.93% to 3.48% (Table 4.3). The background level of %DNA in tail in Lingzhi treatment remains similar with the water treatment after 180 mins post ingestion (Table 4.4). The results of the H_2O_2 challenge version of the comet assay (the ability against oxidative challenge) for pre- and post-ingestion samples are shown in Figure 4.5, 4.6 and Table 4.3, 4.4. Results showed that there was no significant change in resistance to oxidative challenge after single dose of water or Lingzhi. There was ~70% less in %DNA in tail after 180 mins repair in complete medium after both treatments, and the decrease with each treatment was statistically significant ($p<0.01$). However, no significant difference was found between treatments. Results indicate no Lingzhi-associated enhancement of DNA repair of oxidative damage at 3 hours after a single dose (Figure 4.7 and Table 4.5).

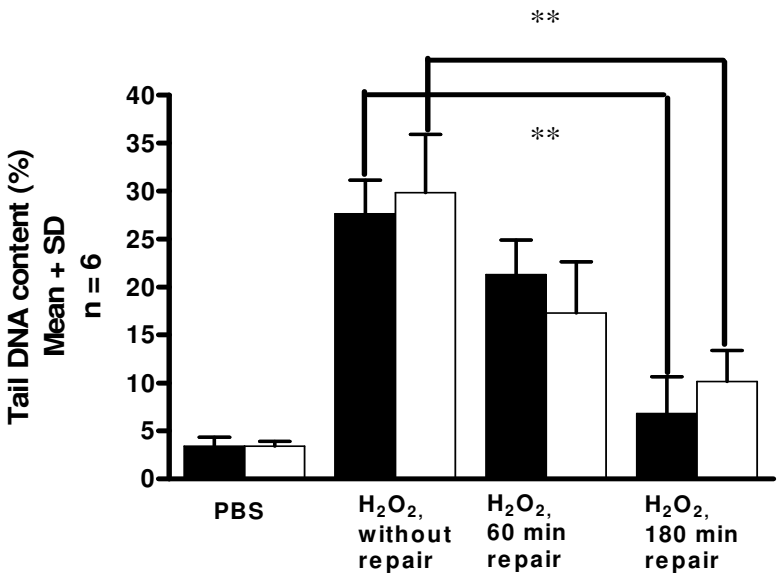


Figure 4.5. The % DNA in comet tail (n = 6; Mean +/- SD) after 60 mins and 180 mins repair (in complete medium) in fasting samples. The solid bar represents pre-Lingzhi treatment and open bar represents pre-water treatment.

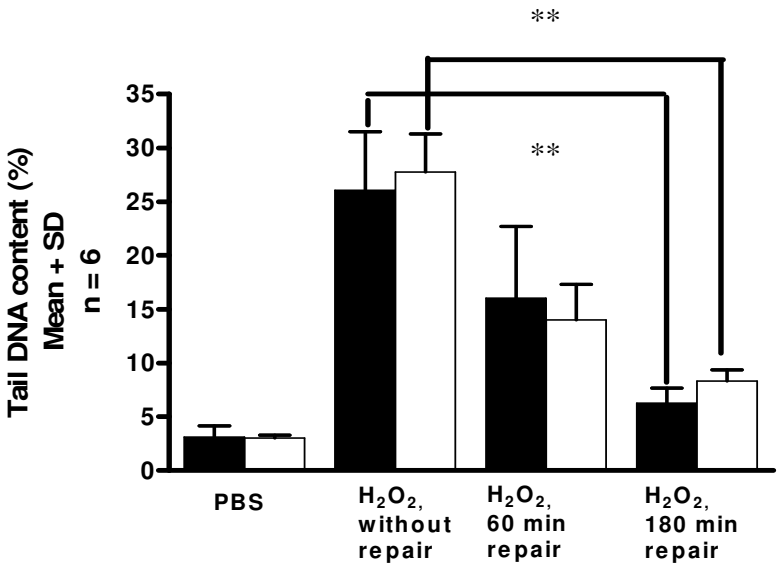


Figure 4.6. The % DNA in comet tail (n = 6; Mean +/- SD) after 60 mins and 180 mins repair (in complete medium) in 180 mins post ingestion samples. The solid bar represents Lingzhi treatment and open bar represents water treatment

Table 4.3. Comet assay results (% DNA in tail; n=6; Mean (SD)) in human lymphocytes collected in fasting state, with or without oxidative challenge with H_2O_2 and allowed to repair in complete culture medium for up to 180 mins.

	0 mins (immediately after challenge)		+60 mins Repair		+180 mins Repair	
	Water treatment	Lingzhi treatment	Water treatment	Lingzhi treatment	Water treatment	Lingzhi treatment
Unstressed cells (PBS)	3.40 (0.52)	3.42 (0.91)	3.47 (1.27)	3.04 (0.70)	3.48 (0.59)	2.93 (0.44)
30μM H_2O_2 stressed cells	29.83 (6.09)	27.64 (3.47)	17.30 (5.32)	21.30 (3.62)	10.17 (3.21)	6.82 (3.82)

Table 4.4. Comet assay results (% DNA in tail; n = 6; Mean (SD)) in human lymphocytes collected after 180 mins post ingestion Lingzhi or water, with or without oxidative challenge with H_2O_2 and allowed to repair in complete culture medium for up to 180 mins.

	0 mins (immediately after challenge)		+60 mins Repair		+180 mins Repair	
	Water treatment	Lingzhi treatment	Water treatment	Lingzhi treatment	Water treatment	Lingzhi treatment
Unstressed cells (PBS)	3.03 (0.29)	3.13 (1.04)	3.90 (0.61)	3.65 (0.66)	3.69 (0.62)	2.97 (0.77)
30μM H_2O_2 stressed cells	27.77 (3.52)	26.05 (5.48)	14.00 (3.29)	16.05 (6.63)	8.33 (1.04)	6.27 (1.40)

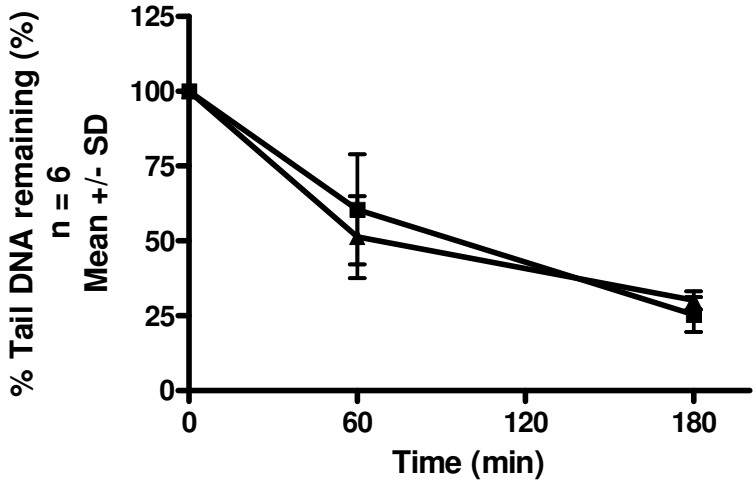


Figure 4.7. The % DNA remaining in tail in human lymphocytes collected after 180 mins post-ingestion and allowed to repair in complete culture medium for up to 180 mins. The square represents Lingzhi treatment and triangle represents water treatment.

Table 4.5. The % DNA remaining in tail in human lymphocytes collected pre and 180 mins post-ingestion of Lingzhi or water and allowed to repair in complete culture medium for up to 180 mins (n = 6; Mean (SD)).

	+60 mins		+180 mins	
	Water control group	Linzghi group	Water control group	Linzghi group
Fasting	58.8 (19.1)	77.5 (12.5)	35.9 (14.6)	25.5 (14.7)
180 mins post ingestion	51.3 (13.7)	60.5 (18.4)	30.1 (3.1)	25.4 (5.8)

Discussion

In a previous study by our team, a single high dose of Lingzhi extract (3.3g) increased plasma antioxidant level significantly (Wachtel-Galor *et al.*, 2004b). In that study, the background DNA level and DNA damage induced by H_2O_2 did not show significant change. However, lymphocytic DNA repair capacity was not assessed. Therefore, in this study, a single high dose acute post ingestion effect of Lingzhi on DNA repair capacity in human lymphocytes was investigated. Results showed that the plasma FRAP value increased quickly after Lingzhi ingestion. This is similar to the result seen in our previous study, however the responses varied quite widely and the increase was not statistically significant overall. Furthermore, Lingzhi did not show any protective effect on background DNA level or on resistance to H_2O_2 challenge. This is also consistent with our previous study results (Wachtel-Galor *et al.*, 2004b). Some published cell culture studies have reported that Linzghi can increase resistance to oxidative damage (Lee *et al.*, 2001; Lai *et al.*, 2006). However, precaution should be taken when interpreting results from *in vitro* study. Cell culture medium usually lacks antioxidants, such as tocopherols and ascorbic acid. Furthermore, most human cells are expose to low oxygen conditions (1-10mm Hg) under normal physiological conditions, whereas in cell culture cells are exposed to ~150mm Hg oxygen tension (Halliwell, 2003). The beneficial effects of Lingzhi observed in the studies of Lee *et al.* (2001) and Lai *et al.*(2006) may be due to replenishment of antioxidants, and these effects may not be seen in cells under physiological conditions, such as within the body. Human supplementation

trials are is needed to prevent over-interpretation of beneficial effects of Lingzhi shown in *in vitro* study.

In this current study, Lingzhi did not show an effect on DNA repair in cells collected shortly after ingestion. The disappearance of strand breaks after oxidation-induced challenge was not any faster in samples collected 180 mins after Lingzhi ingestion as compared to after water ingestion. Although the plasma FRAP value increased rapidly after ingestion, this does not necessarily indicate that intracellular antioxidant defence was increased at the same time. The peak concentration of antioxidants in cell may be reached after the peak in plasma, or Lingzhi antioxidants in plasma might not enter cells. In this study, lymphocytes were collected at 180 mins post ingestion. It is possible that Lingzhi bioactive components were still not available intracellularly by that time, and time point for harvesting lymphocytes beyond 180 mins post ingestion may be needed to demonstrate the DNA repair effect. The delay between peak plasma levels (antioxidant level) and peak cellular response in terms of DNA protection was observed by Collins *et al.* The maximum ability of human lymphocyte against H_2O_2 challenge was seen after 8 hrs post ingestion of single dose of kiwifruit juice (500 ml) (Collins *et al.*, 2001c), and the effect remained significant after 24 hrs ingestion ($P< 0.005$).

The major limitation of this study is the sample size. The number recruited was small, but is enough for a pilot study to obtain preliminary results. Post-hoc power calculation showed that this sample size is able to detect a true difference of

1.43 SD at 80% power and 5% significance level. From the results obtained in this study, an SD of 3.21 was found in lymphocyte incubated up to 180 mins after H_2O_2 challenge. This means that a change in 4.59% of %DNA in tail could be detected. However, a larger study is needed to be able to detect smaller effects that might be induced by Lingzhi, especially in healthy subjects. Another favourable approach might be to investigate effects on subjects under increased oxidative stress or with decreased DNA repair capacity. DNA repair is a complex process which involves different enzymes responsible for different steps (Cleaver & Crowley, 2002; Christmann *et al.*, 2003). In this study, only the overall DNA repair capacity was assessed. Therefore, another limitation of this study is we cannot tell which particular pathway that Lingzhi may act on, if any.

In conclusion, results of this part of study showed Lingzhi can increase plasma antioxidant level rapidly, but changes were small and highly variable and did not reach statistical significance. Lingzhi had no detectable effect on baseline DNA level, resistance to H_2O_2 challenge or on timed DNA repair of induced damage in the 3h post-ingestion samples. This may be due to the small sample size, or the effect is not observable in healthy young adults. A larger study in population at higher risk group may be needed to observe beneficial effects of Lingzhi. It is also possible that Lingzhi only demonstrates DNA repair effects after a longer supplementation period since time is needed for DNA repair enzymes to be upregulated. However, it must be noted that Lingzhi may have no effect on DNA repair.

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

Antioxidant power and DNA repair effects of Lingzhi or Reishi medicinal mushroom, *Ganoderma lucidum* (W.Curt.:Fr.)P. Karst. (Aphyllophoromycetideae) in human acute post-ingestion study. *Int J Medicinal Mushrooms* 2010.

(with Wachtel-Galor S, Choi SW, Benzie IFF)

Chapter 5

EFFECT OF GREEN TEA ON DNA, WITH MAIN FOCUS ON URINE 8-OXO-7,8-DIHYDRO-2'DEOXYGUANOSINE EXCRETION IN HEALTHY ADULTS: AN EXPLORATORY STUDY IN ASSOCIATION WITH Fpg-ASSISTED COMET ASSAY RESULTS

Introduction

Tea is rich in polyphenolic antioxidants and is suggested to have many health benefits (Williamson & Manach, 2005; Khan & Mukhtar, 2007; Yang *et al.*, 2009). Tea is brewed from the leaves of the plant *Camellia sinensis* and is the second most consumed beverage worldwide, after water (Weisburger and Chung, 2002). There are three main types of tea, which are green tea (unfermented), black tea (fermented) and oolong tea (semi-fermented) (Shukla, 2007). Black tea is most commonly consumed in Western countries, and is produced by allowing crushed tea leaves to wither for a few hours during which time the activated polyphenol oxidase enzyme in the leaves changes the nature of the polyphenol content of the tea leaves. Green tea is most commonly consumed in Asian and African countries, and is produced by steaming fresh leaves soon after being picked, and drying them. The steaming inactivates the enzyme and prevents oxidation of the polyphenols in the leaves. Oolong tea is commonly consumed in southern China, and is produced by

partial fermentation, therefore its composition is intermediate between green tea and black tea (Khan & Mukhtar, 2008).

Polyphenolic compounds are commonly found in plants and have been suggested to lower the risk of cancer, diabetes and cardiovascular disease (Weisburger & Chung, 2002; Khan & Mukhtar, 2007). Around 30 % dry weight of green tea is made up of a group of polyphenolic compounds called catechins. The catechins in green tea are epigallocatechin-3-gallate (EGCG) (the major type), epigallocatechin (EGC), epicatechin (EC) and epicatechin-3-gallate (ECG) (Khan & Mukhtar, 2007). The potential health benefits of green tea may be associated with the antioxidant properties of these polyphenolic compounds. However, the bioavailability of polyphenolic compounds is poor; only a small portion is absorbed and is measurable in plasma. A study of eight healthy adults showed the plasma maximal concentration of EGCG was ~ 78 ng/ml (<0.2 µmol/l) at around 1.5 hrs after oral ingestion of green tea solids (20 mg/kg body weight) dissolved in hot water (Lee *et al.*, 2010). However, even though the bioavailability of tea polyphenols is poor, our previous study showed that the plasma antioxidant content increased by ~ 4 % and peaked at ~40 mins after intake of green tea, returning to baseline within 3 hrs (Benzie *et al.*, 1999). The specific component of green tea that caused this increase was not evaluated. However, regular intake of green tea may, through an accumulation of antioxidant or other components or effects, have beneficial effects on health. Indeed, it is generally believed that green tea has beneficial effects on health. However, results from various studies on green tea are

inconclusive (Erba *et al.*, 2005; Henning *et al.*, 2005; Møller & Loft, 2006). Furthermore, based on our best knowledge there are few human trials that have investigated the genoprotective effects of green tea.

Protection and repair of DNA damage is important. If oxidatively modified DNA remains unrepaired before replication, accumulated modified DNA in daughter cells may initiate carcinogenic change (Halliwell & Gutteridge, 2007; Collins, 2009). In measuring DNA damage, the comet assay is a simple, sensitive and widely used method for measuring damage (as single strand breaks) at the cellular level (Wong *et al.*, 2005; Dusinská & Collins, 2008). Due to the mutagenic ability of G:C → T:A transition mutation, 8-oxodG, the DNA lesion that leads to such transition , is the most extensively studied oxidation-induced DNA lesion (Lunec *et al.*, 2002; Cooke *et al.*, 2009). There is no direct evidence that increasing 8-oxodG level in DNA increases cancer risk. However, the level of 8-oxodG is elevated in breast, colon, kidney and lung cancer tissues (reviewed by Cooke *et al.*, 2003). Some workers have measured the effect of green tea on 8-oxodG in lymphocytes (Henning *et al.*, 2005). In a single dose study (Henning *et al.*, 2005) of 20 healthy adults who took green tea extract, lymphocyte 8-oxodG, measured by HPLC and expressed as a ratio to total lymphocyte dG (deoxyguanosine), did not change over the 24 hrs following ingestion of the extract. In a study of smokers and non-smokers, green tea consumption was reported to decrease 8-oxodG in white cells and in urine (Klaunig *et al.*, 1999). However, measuring 8-oxodG in cells is problematical and the HPLC-ECD method used in some studies is less sensitive

than LC-MS/MS (Gedik & Collins, 2005; Lee *et al.*, 2010). Because of difficulties in measuring cellular 8-oxodG, its measurement in urine has attracted attention. Urine needs no extraction, and 8-oxodG in urine is not directly affected by diet, cell death and cell turnover, it is stable in urine (up to 2 years at -80^{o}C), and does not vary systematically during the day, nor does it vary significantly day to day within a group (though biological variation is wide) (Matsumoto *et al.*, 2008; Cooke *et al.*, 2009; Lee *et al.*, 2010).

Many studies (reviewed by Møller & Loft, 2006) in recent years have used urine 8-oxodG as biomarker of DNA damage (and protection), the reasoning being that oxidized dG within DNA is repaired by BER and the oxidized base is then excreted into the urine. In the planning of this current study our intention was to use urine 8-oxodG as another test (in addition to the comet assay) of DNA damage according to this understanding at the time. However, it is becoming clear that the source of 8-oxodG is not dG from cellular DNA, but is most likely from oxidized bases within the nucleotide pool (Cooke *et al.*, 2008). Therefore, urine 8-oxodG should be regarded as a biomarker of whole body oxidative stress rather than a biomarker of DNA damage, as it was previously thought to be. Nonetheless, this green tea supplementation trial offered an excellent opportunity to explore 8-oxodG as a biomarker of whole body oxidative stress and its relationship to DNA damage by investigating:

a) the effect of antioxidant rich supplement (green tea) on this biomarker of whole body oxidative stress as well as on Fpg labile sites in lymphyocytic DNA.

b) the relationship between Fpg comet assay data on lymphocytes and urine 8-oxodG excretion

The relationship between Fpg labile sites and urine 8-oxodG has not to our knowledge been previously studied. There are a few studies that have investigated the effect of green tea on urine 8-oxodG or DNA damage using the comet assay. In an uncontrolled acute ingestion study (Morley *et al.*, 2005) 10 healthy adults ingested 540 ml of green tea and peripheral lymphocytes collected before and 40 mins after ingestion were irradiated *in vitro* with UVA/Visible light. The standard alkaline comet assay was used to investigate single strand breaks and the study reported significantly less DNA damage after tea compared to before tea. However, tea polyphenols absorb UV light (Wei *et al.*, 2009) and the protection may have been due to a 'sunscreen' effect rather than an antioxidant effect of the tea. There is only one published human supplementation trial with green tea that used the comet assay (Erba *et al.*, 2005). In a study of 12 healthy adults (only 10 of whom completed the trial) who took 2 cups of green tea per day for 42 days, significantly less DNA damage (using the standard alkaline version of the comet assay) was seen in lymphocytes exposed *in vitro* to Fe^{2+} (Erba *et al.*, 2005). This study suggests that

green tea ingestion may increase resistance of DNA to oxidant challenge. However, the study was small and no basal oxidation-induced damage was measured.

In previous human studies of effects of green tea on urine 8-oxodG, results are inconclusive (Young *et al.*, 2002; Hakim *et al.*, 2003; Luo *et al.*, 2006). Luo *et al.* showed that after 3 months' green tea polyphenols (500mg or 1000 mg/day) consumption, the urine 8-oxodG detected by HPLC- ECD was significantly lowered in 42 (consuming 500 mg/day) and 41 (consuming 1000mg/day) subjects at high risk of liver cancer. However, no significant change in urine 8-oxodG was observed in any groups after 1 month green tea polyphenols consumption (Luo *et al.*, 2006). Hakim *et al.* showed that after 4 months' decaffeinated green tea (4 cups/day, supplying ~294 mg catechins) consumption, urine 8-oxodG detected by HPLC-ECD was significantly lowered in 42 smokers (Hakim *et al.*, 2003). However, a crossover study in which 8 smokers and 8 non-smokers were given green tea extract incorporated into meat patties (18.6 mg catechins/day) for 3 weeks showed no changes in urine 8-oxodG detected by HPLC- ECD (Young *et al.*, 2002).

In summary, studies of effects of green tea supplementation on oxidation induced DNA damage and urine 8-oxodG in humans are few and results are contradictory. Furthermore, no previous study has investigated the relationship between lymphocytic Fpg labile sites and urine 8-oxodG or used both in a green tea supplementation trial. Therefore, the aims of the current study were to investigate:

i) the effect of green tea supplementation on whole body oxidative stress (using urine 8-oxodG as the biomarker) and oxidation-induced basal DNA damage (using the Fpg-assisted comet assay)

and

ii) the relationship between urine 8-oxodG and Fpg-comet assay results in healthy subjects.

Finally, to be able to generalize the results more to green tea overall, two types of green tea were used in this controlled supplementation trial. The teas selected were Loongjin and Screw-shaped tea. These are both popular and commonly consumed teas in Hong Kong.

Materials And Methods

Chemicals And Reagents

The 8-oxodG (Thin-Layer Chromatography grade) and ammonium acetate (Ultra grade) were from Sigma (St. Louis MO, USA); acetic acid (AnalaR) and acetonitrile (HiperSolv for HPLC) were from BDH Laboratory Supplies (Poole, Dorset, UK). Reagents for the comet assay were as described in Chapter 3.

Supplementation Scheme

This was a single blinded, multiple cross-over intervention trial with 18 healthy subjects (9 male, 9 female), between 35 and 50 years old [mean (SD) 42.6 (3.6)]. Written informed consent was obtained from each subject before the study

began. This study was approved by The Human Subjects Ethics Subcommittee of The Hong Kong Polytechnic University (Appendix 2) Subjects were allocated on a non-selective basis, but stratified for number, to 4 weeks' supplementation with (i) 300ml/day of freshly prepared 1.0% w/v Loongjin tea (1.5 g tea leaves in 150 ml hot water taken twice daily), or (ii) 300 ml/day of freshly prepared 1.0% w/v of Screw-shaped (1.5 g tea leaves in 150 ml hot water taken twice daily) or (iii) 150 ml hot water, taken twice daily. Tea was prepared as described below. Each subject was requested to ingest the 150 ml dose of green tea or water in the morning and evening during the intervention period. After 6-weeks' washout, the procedure was repeated with subjects crossed-over onto one of the other two treatments. The procedure was repeated after a second 6-weeks' washout, during which subjects took the final treatment. Figure 6.1 gives an outline of the overall supplementation scheme. All subjects took all three treatments. Treatments were coded and this investigator did not know which treatment was given each time. Codes were broken after data were collected.

Preparation Of Tea

Both types of green teas were kindly supplied by Ying Kee Tea House, Hong Kong. Tea bags, which contained 1.5 g green tea added to commercially available empty tea bags and specially prepared for this study by another research team member. Green tea with final concentration of 1% w/v was prepared by adding 150 mL water to one 1.5 g green tea bag in a cup: the hot water had been allowed to stand for 5 mins after boiling before adding to the cup with the green tea bag. The

tea bag was removed after the tea had brewed for 3 mins, and the tea was ingested within 20 mins. Sixty tea bags and a cup marked at 150ml level were provided to each subject and 85 % compliance was regarded as acceptable. During the 4 weeks' intervention period, 56 tea bags should have been used. The compliance was checked by regular contact with volunteers by our team members and by counting returned tea bags at the end of each intervention period. For 85 % compliance, at least 48 tea bags had to be used. All subjects returning with more than 12 tea bags were considered as non-compliant. One subject returned 24 unconsumed tea bags at the end of the second intervention period. All data from that subject were excluded.

Sample Collection

Pre and post each supplementation period, spot, fasting urine samples were collected into a clean container without preservative. On arrival at the lab, which was within 2 hrs of collection, urine was centrifuged (3,500 rpm for 15 mins) and the supernatant was aliquoted. One aliquot was used for measurement of creatinine (on day of collection) and other aliquots were stored at -80^{o}C until batch tested for 8-oxodG, which was within 1 month of collection. Fasting venous blood samples were also collected pre and post each treatment and put into commercial heparin tubes. There were six urine samples collected from each volunteer during the course of the study. The samples were coded and investigator was blinded during the study period. The urine samples were treated as described in detail below. Lymphocytes from the blood samples were harvested and the Fpg-assisted comet assay was performed on these as described in detailed in Chapter 3. In this part of the study,

this author performed the 8-oxodG measurements, while another member of our team performed the comet assay.

Urine Creatinine Measurement

Creatinine in urine was measured by the alkaline picrate method using a test kit from BioSystems (Barcelona, Spain) on a Cobas Fara analyzer (Roche Diagnostic Ltd, Basel, Switzerland) Lyphocheck 1 and 2 were used as controls with CVs between and within run of <3%, and multicalibrator (all from Bio-Rad, Irvine, CA, USA) was used as standard. Creatinine was used to normalize urine 8-oxodG concentration, due to the differences in urine concentration of each sample.

Urine 8-oxodG Measurement By LC-MS/MS

The procedure used was that of Lee *et al.*, 2010 and is described in detail below.

Preparation Of Pooled Urine, Controls, Calibrators And Samples

Pooled urine for controls was obtained from 5 healthy human subjects. Some of the pooled urine was stored for use as a matrix for preparation of 8-oxodG calibrators. Some was spiked with pure 8-oxodG to prepare low (14.1 nmol/l) and middle (56.5 nmol/l) levels of urine 8-oxodG control samples. For calibration, a stock solution of 8-oxodG was prepared by dissolving around 0.3 mg of 8-oxodG in 1 ml Milli-Q water. From this stock solution, a diluted intermediate stock solution was made up and the exact concentration was determined by measuring the

absorbance at 248 nm by UV spectrophotometer (Beckman UV spectrophotometer DU 730; Beckman Coulter, Inc., Fullerton, CA, USA) and divided by the extinction coefficient of 12,300 (Huang *et al.,* 2001). Both the stock (~0.2 µmol/ml) and the intermediate stock (~0.05 µmol/ml) were stored at -80 °C. A weekly working solution (of known concentration, and generally ~1.13 nmol/ml) of 8-oxodG in Mill-Q water was prepared from the diluted intermediate and stored at 4 °C. On the day of analysis, aliquots of urine samples (pooled urine for matrix for daily calibrators, and test urine samples) were thawed, mixed vigorously and sonicated for 1 min. A clear supernatant was obtained after centrifugation at 10,000 rpm for 5 mins. From the weekly working solution of 8-oxodG, various concentrations of daily working calibrators (7.1, 14.1, 28.2, 56.5 nmol/l) were prepared by dilution with the clear supernatant of the pooled urine. Finally, 25 µl of calibrator, control (low and middle level 8-oxodG spiked urine) and samples were injected into the HPLC-MS/MS system.

Chromatographic Conditions

The HPLC system used included an Aligent 1100 series HLPC unit with quaternary pump (G1311A), micro vacuum degasser (G1379A) and non-thermostatted well-plate autosampler (G1313A). (Agilent Technologies Inc., Waldbronn, Germany). Separation was performed on Merck Chromolith Performance RP-18e column (4.6 mm ID x 100 mm L, particle size: 2 µm x 13 nm pore) and a Phenomenex guard cartridge (C_{18}, 3 mm ID x 4 mm L) (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 96.4 % v/v ammonium acetate

(10 mmol/l, adjusted to pH 4.3 by adding acetic acid) and 3.6 % v/v acetonitrile, which was filtered and degassed through a Millipore membrane (type GV, pore size 0.22 μm, Millipore Corp.) before use. The flow rate was set to 2 speeds; 0.5 ml/min for 0-10 mins, followed by a wash period of 6.0 mins at 2 ml/min. The increased flow rate was used to clean the column between each sample. An integrated diverting valve installed at the entrance to the spectrometer was used to deliver the 8-oxodG fraction eluting from the HPLC system into the mass spectrometer (7.5 – 10 mins eluate). The calibrator (28.2 nmol/l) was injected every 15 samples to monitor elution from the LC column to ensure that the correct eluate fraction was being introduced into the detector during long runs. The columns worked under room temperature. The elution time was pre-determined during method set-up by injecting pure 8-oxodG standard into the LC system with detection by UV at 260nm.

Mass Spectroscopy Conditions

Detection of 8-oxodG was performed by a 3200 QTRAP mass spectrometer from Applied Biosystems/MDS Sciex (MDS Inc., Concord, ON, Canada), under positive electrospray ionization mode with a TurboIonSpray® source. The spectrometer condition was optimized by infusion of an 8-oxodG standard in water (2.0 μg/ml, M.W = 283.2) by using an integrated syringe pump (flow rate at 5 μl/ min). The settings were established by multiple reaction monitoring (MRM) detection. The m/z of precursor ($[M+H]^{1+}$) and product ion was 284 and 168 respectively. The source parameters were set as follows: curtain gas (nitrogen), 12.0 psi; collision gas (nitrogen), medium; ionspray voltage, 5500 V; temperature, 375°C;

ion source gas (air) 1, 11.0 psi; ion source gas (air) 2, 32.0 psi; and interface heater on. The parameters for the optimized compound were set as follows: declustering potential, 26.0 V; entrance potential 3.5 V; collision energy, 19.0 eV; and, collision cell exit potential, 3.9 V. All data were acquired and manually processed by the Analyst® Software 1.4.2 (MDS Inc., Concord, ON, Canada).

Precision And Recovery

Within-day precision was assessed by 4 calibration curves (using calibrators of 0, 7.1, 28.2 and 56.5 nmol/l in matrix urine as described above) performed in one day. Between-day precision was assessed by 4 calibration curve (using calibrators of 0, 7.1, 28.2 and 56.5 nmol/l in matrix urine as described above) performed over 4 different days. For estimation of recovery, two sets of 8-oxodG -spiked solutions (7.1, 28.2 and 56.5 nmol/l, each in 4 replicates) were prepared in water and pooled urine. The relative recovery in urine matrix (%) was calculated by "recovery of 8-oxodG added to urine / recovery of 8-oxodG added to water x 100%".

Limit Of Detection (LOD)

The absolute limit of detection (LOD) was determined by injection of diluted solutions of standard in water in decreasing concentrations. The LOD is the lowest concentration of analyte that can be distinguished from background noise in an instrument, and therefore is a detection limit in a clean matrix. The LOD in the current study was identified as the concentration giving a signal to noise ratio equal to 3. The 'true' LOD is the lowest concentration of analyte that can be distinguished

in the urine matrix. The 'true' LOD in the current study was determined from 4 calibration curves in urine matrix (y = mx + b) after subtracting the 8-oxodG urine basal peak area. The true LOD was calculated as 2 x SD of intercept b, then divided by slope, m.

Formamidopyrimidine-DNA Glycosylase (Fpg)-Assisted Comet Assay For Oxidation-Induced Damage To DNA

The method used to investigate basal oxidation-induced DNA damage was the Fpg-assisted comet assay. This method was described in detail in Chapter 3. To recap briefly, Fpg enzyme solution was added to each gel containing embedded cells after the lysis step. Fpg enzyme creates breaks at sites of oxidized purines and ring-opened purines (8-oxoGua is the major substrate), and the Fpg-assisted comet assay increases the sensitivity 4-fold compared to the standard alkaline version and is more specific to detection of oxidation-induced DNA lesions (Collins, 2009). In this part of the study the Fpg-assisted comet assay was performed by another member of our research group, and data were retrieved by this investigator.

Statistical Analysis

The Kruskal-Wallis test with Dunnet's post test was used to investigate differences in responses to each tea compared to water (control treatment). Spearman's correlation was used to investigate the relationship of urine 8-oxodG with Fpg-assisted comet assay data (at one matched time-point only). Prism 4.0 (GraphPad Software, San Diego, CA, USA) was used. $P < 0.05$ is considered as

statistically significant. The key variable in this study was the %DNA in comet tail after Fpg treatment. Our previous work shows that the SD in Fpg-assisted comet is 3.5%. Therefore to detect a difference of ≥3.0% in the mean %DNA in comet tail with power of 84% and at the 5% significance level, 14 subjects in a cross-over trial were needed (by PS-Power and sample size calculation V3.0.4). For 8-oxodG we had no local data as the LC-MS/MS equipment had only recently been installed in our laboratory when the study was planned. Also published data on 8-oxodG were confusing due to different units and non-standardization to creatinine. We therefore performed a preliminary power calculation in relation to delta in SD units, and found that 18 pairs (the number selected for the study) could detect a true difference in the mean of 0.7 SD at 80% power and 5% significance level. As data on healthy Chinese subjects were gathered by our group, an SD of 0.6 nmol 8-oxodG per mmol of creatinine was found, meaning that a true difference in 8-oxodG of 0.42 nmol/mmol creatinine could be detected. Due to technical problems with some samples, and non-compliance by one subject, only 14 complete data sets for urine 8-oxodG were available. In a post-hoc power calculation with n = 14, the true difference in urine 8-oxodG that can be detected at 80% power and $P<0.05$ is 0.48 nmol/mmol creatinine.

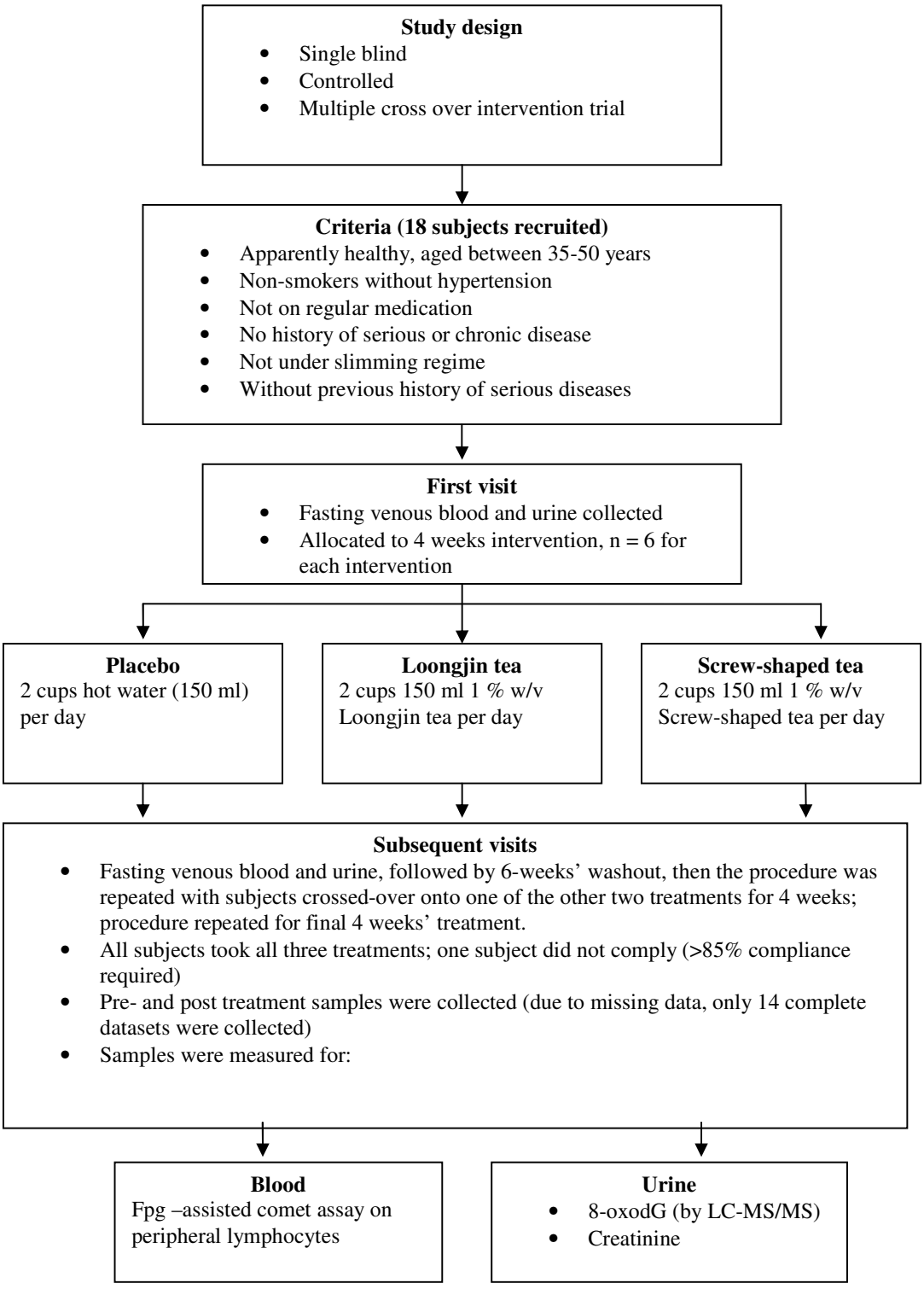


Figure 5.1. Outline of study protocol.

Results

a) Method Characteristics

Linearity, Precision, Accuracy And Recovery

A typical mass spectrum for 8-oxodG in urine matrix are shown in Figure 5.2. The calibration curve was linear in the range 0-113 nmol/l, the highest level tested in the current study (r^2 >0.99; Figure 5.3). The CVs for the within-day and between-day precision over the different concentrations tested ranged from 1.2 % to 3.7 % (Table 5.1) and 3.6% to 5.5%, respectively (Table 5.2). The recovery of 8-oxodG in this method ranged from 101% to 103% at three 8-oxodG concentrations ranging from 7-56 nmol/l (Table 5.3). It can be seen that the recovery in the test matrix (urine) was very similar (and approaching 100%) to the recovery in water, indicating no matrix effect or ion suppression in the fraction of interest. The true and absolute LODs for 8-oxodG in this method were 2.32 nmol/l and 1.8 nmol/l, respectively.

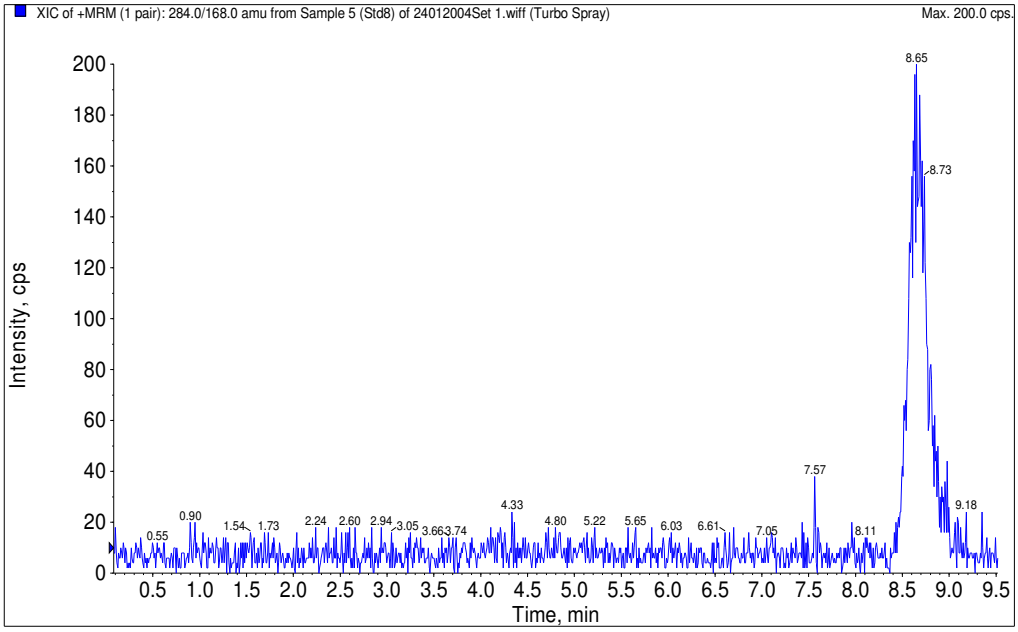


Figure 5.2. Typical mass spectrum of urine 8-oxodG (m/z 284-168amu).

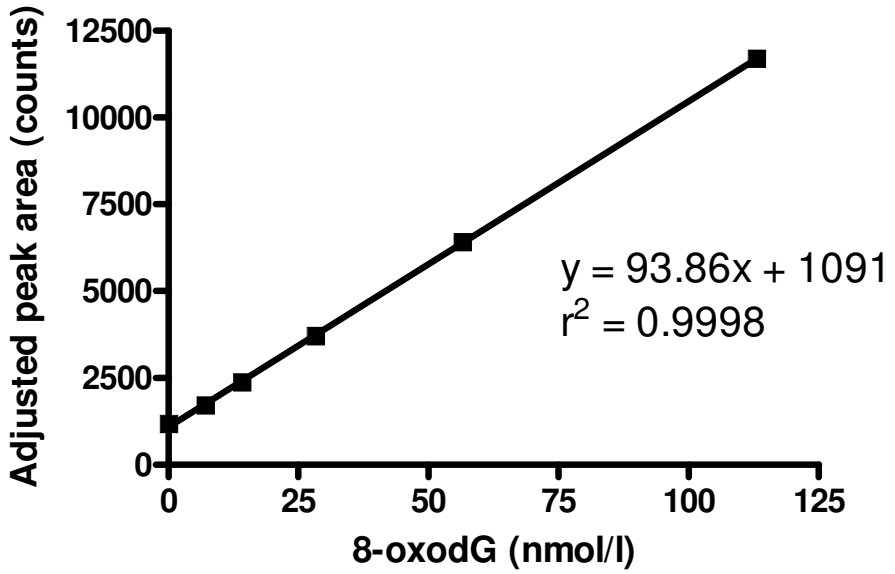


Figure 5.3. Typical calibration curve of urine 8-oxodG.

Table 5.1. Within-day precision of urine 8-oxodG (n = 4 at each of the concentrations).

Amount of 8-oxodG spiked into pooled urine (nmol/l)	Mean (SD) of measured values (nmol/l)	CV (%)
7.1	30.06 (1.15)	3.70
28.2	50.99 (2.97)	5.83
56.5	80.39 (0.96)	1.20

Table 5.2. Between-day precision of urine 8-oxodG (n = 4 at each of the concentrations).

Amount of 8-oxodG spiked into pooled urine (nmol/l)	Mean (SD) of measured values (nmol/l)	CV (%)
7.1	19.65 (1.09)	5.53
28.2	40.96 (2.02)	4.94
56.5	71.84 (2.61)	3.64

Table 5.3. Recovery study of urine 8-oxodG (n = 4 at each of the concentrations) spiked into water and in pooled urine

Expected amount of 8-oxodG from the spike (nmol/L)	Recovery of 8-oxodG added to Water (Mean % Recovery, n=4)	Recovery of 8-oxodG added to Urine (Mean % Recovery, n=4)	Relative Recovery in Urine Matrix (%)
7.1	102.8	104.2	101.4
28.2	105.7	108.6	102.7
56.5	102.7	106.0	103.2

b) Results Of The Green Tea Intervention Trial: 8-oxodG In Urine

As noted above, data from one subject were excluded due to non-compliance. In addition, some samples had 8-oxodG values under the 'true' LOD. These values were excluded from analysis. Therefore, only 14 complete data sets were available from the supplementation trial. The results of urine 8-oxodG for pre- and post-supplementation samples are shown in Figure 5.4 and Table 5.4. The urine 8-oxodG concentrations varied from ~0.3 to 3.3 nmol/mmol creatinine overall, and the distribution was skewed. Values decreased by an average of ~4% to 5% after supplementation with Loongjin tea and Screw-shaped tea compared to a 2% increase after water treatment. However, none of these within- treatment differences reached statistical significance. When the responses to each tea (the post-pre difference) were compared to the responses to water (control) in non-parametric paired analysis, no statistically significant differences were seen (Figure 5.5 and Table 5.4). The individual pre and post results of the 14 subjects for whom complete

data sets were available are shown in Figure 5.6 for water, Figure 5.7 for Loongjin, and Figure 5.8 for Screw-shaped tea. It can be seen that the individual variation in urine 8-oxodG with all treatments was quite high.

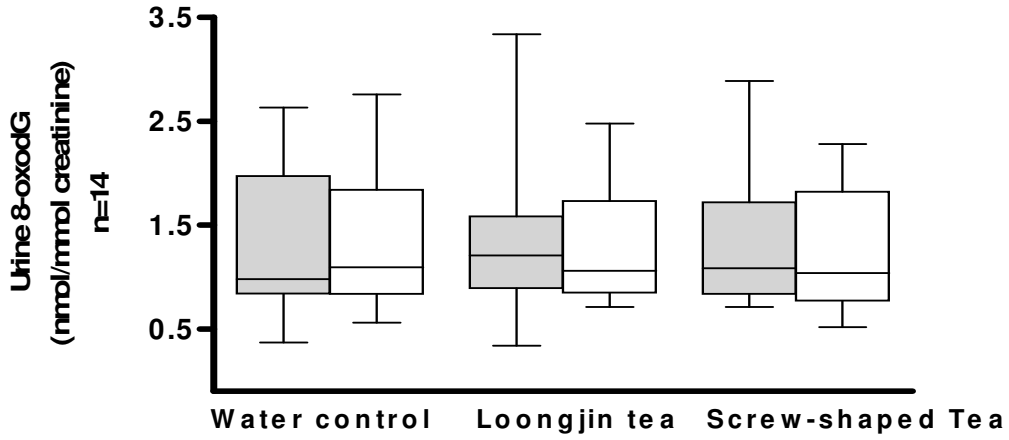


Figure 5.4. Creatinine-standardized urine 8-oxodG concentrations before (shaded bars) and after (open bars) 4 weeks' green tea or water supplementation; results are median with interquartile ranges and lowest and highest values; n = 14.

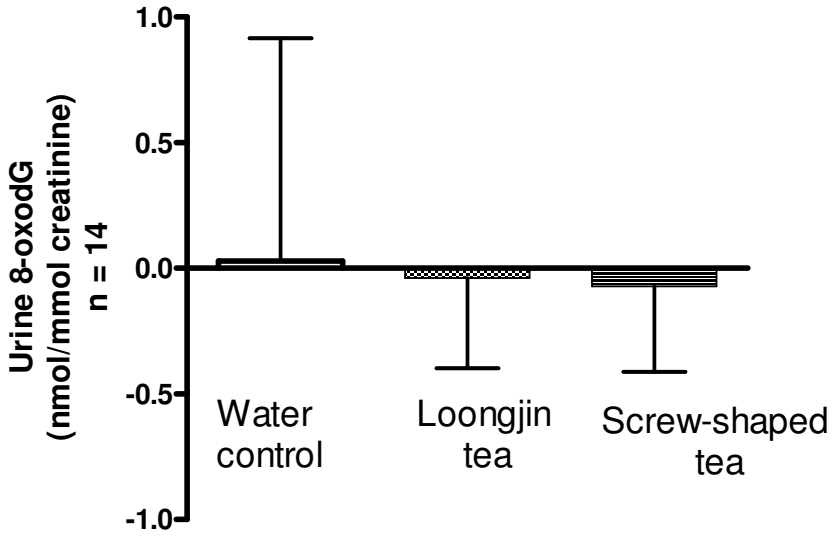


Figure 5.5. Change in creatinine-standardized urine 8-oxodG concentrations after 4 weeks' supplementation with water, Loongjin or Screw-shaped tea. Results are Mean+SD post-pre differences, n=14 for each treatment.

Table 5.4 Summary of urine 8-oxodG results (nmol/mmol creatinine) in 14 apparently healthy Chinese subjects.

	Water		**Loongjin tea**		**Screw-shaped tea**	
	Pre	Post	Pre	Post	Pre	Post
Range	0.37 to 2.63	0.56 to 2.76	0.34 to 3.34	0.71 to 2.48	0.71 to 2.89	0.52 to 2.28
Median	0.98	1.10	1.21	1.06	1.09	1.04
Mean (SD)	1.32 (0.66)	1.35 (0.70)	1.32 (0.72)	1.28 (0.52)	1.29 (0.62)	1.22 (0.56)
95% CI of mean	0.94 to 1.70	0.94 to 1.75	0.90 to 1.74	0.98 to 1.58	0.93 to 1.65	0.90 to 1.54

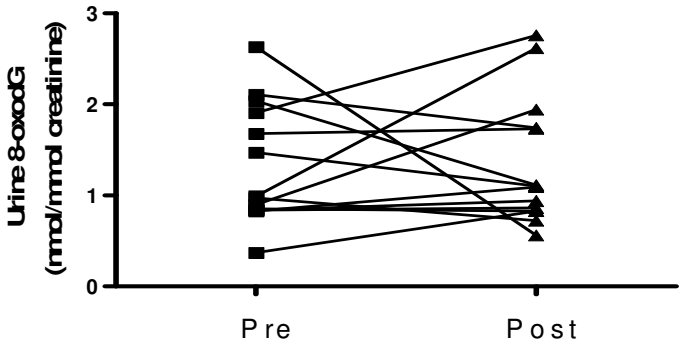


Figure 5.6. Individual results showing changes in urine 8-oxodG (nmol/mmol creatinine) after 4 weeks' supplementation with water (n = 14).

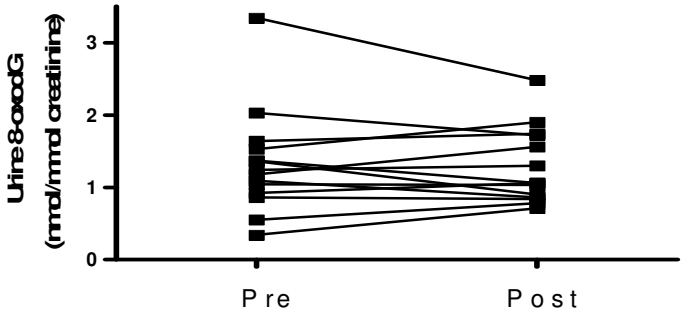


Figure 5.7. Individual results showing changes in urine 8-oxodG (nmol/mmol creatinine) after 4 weeks' supplementation with Loongjin (n = 14).

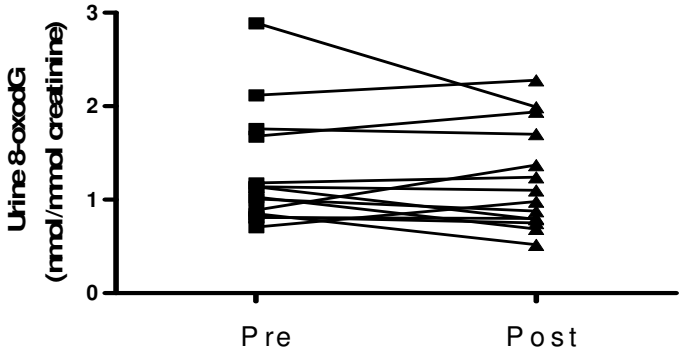


Figure 5.8. Individual results showing changes in urine 8-oxodG (nmol/mmol creatinine) after 4 weeks' supplementation with Screw-shaped tea (n = 14).

The results of the Fpg-linked comet assay (basal DNA damage) for pre- and post- supplementation samples are shown in Table 5.5 (only 13 complete data sets were available). There was ~30% less Fpg labile sites in cells collected post-supplementation with each type of tea, and the decrease with each type of tea was statistically significant ($P<0.05$) when compared to the post – pre supplementation difference after water. Both teas gave similar effects. No significant correlation (using Spearman's rank correlation) was seen between urine 8-oxodG and lymphocytic DNA Fpg labile sites after 4 weeks' supplementation with each kind of tea (Figures 5.9 and 5.10).

Table 5.5. Fpg-assisted comet assay results in lymphocytes collected pre- and post- 4 weeks' supplementation in 13 human subjects in an intervention trial of multiple cross-over design; results are Mean (SD) %DNA in comet tail; *significant (P<0.05) difference compared to response to placebo (water) treatment.

	Water (Control treatment)			**Loongjin tea**			**Screw-shaped tea**		
	Pre	Post	Change	Pre	Post	Change	Pre	Post	Change
Buffer treated cells	5.21 (1.81)	4.84 (1.34)	-0.38 (1.71)	4.19 (0.84)	4.71 (1.78)	0.52 (1.71)	5.15 (0.84)	4.33 (1.09)	-0.82 (1.19)
Fpg treated cells	15.37 (2.74)	15.82 (3.42)	0.46 (4.77)	16.80 (3.12)	11.89 (1.27)	-4.91* (2.97)	16.85 (3.44)	10.75 (4.10)	-6.10* (3.69)

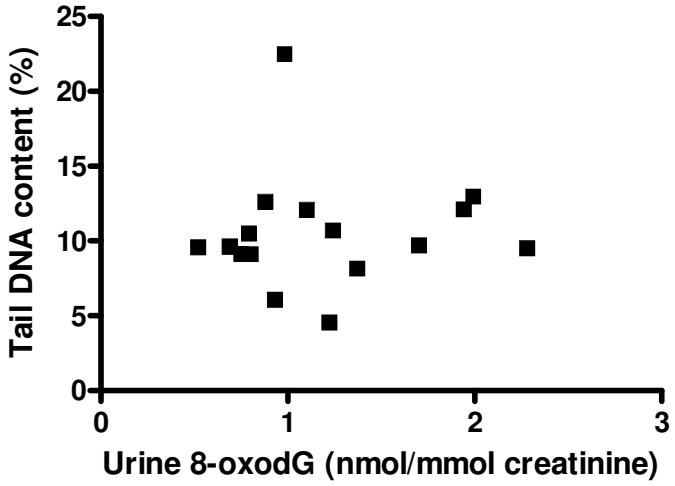


Figure 5.9. No significant correlation seen between urine 8-oxodG concentration and DNA damage (% DNA in tail in Fpg comet assay) in 17 subjects after 4 weeks' supplementation with Screw-shaped tea (Spearman's rho = 0.19, P = 0.47; n = 17).

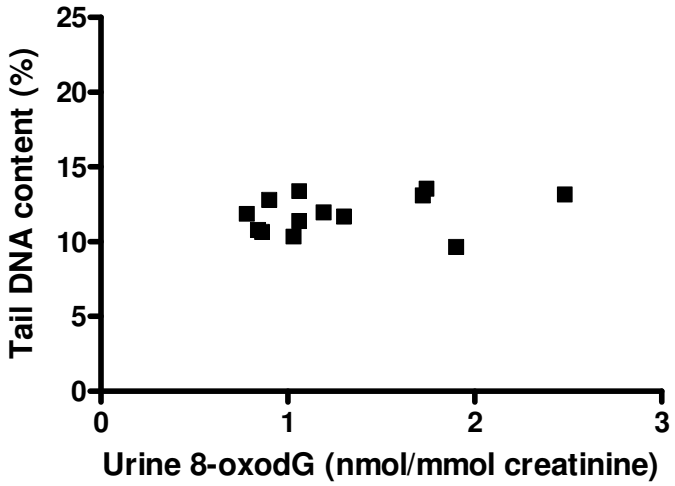


Figure 5.10. No significant correlation seen between urine 8-oxodG concentration and DNA damage (% DNA in tail in Fpg comet assay) in 13 subjects after 4 weeks' supplementation with Loongjin tea (Spearman's rho = 0.33, P = 0.27; n = 13).

Discussion

Oxidatively modified DNA has a wide range of potential effects, such as mutation, apoptosis and altered homeostasis (Halliwell & Gutteridge, 2007; Collins, 2009). There are various antioxidant and repair systems to protect DNA (Cleaver & Crowley, 2002; Cooke *et al.*, 2003; Loft *et al.*, 2006). However, these systems may be insufficient under certain circumstances, such as increased oxidative stress or high exposure to carcinogens. Oxidatively modified DNA, if passed to daughter cells, can accumulate with age, and finally initiate carcinogenesis (Evan & Vousden, 2001; Gorbunova *et al.*, 2007). There is an urgent need to find genoprotective agents for health promotion. Due to the high polyphenol content, green tea is suggested as a potential genoprotective agent (Khan & Mukhtar, 2007; Khan & Mukhtar, 2008; Yang *et al.*, 2009). A few studies investigated the effects of green tea at the DNA level by using urine 8-oxodG as a biomarker (reviewed by Møller & Loft, 2006). However, it has become more clear that the origin of urine 8-oxodG is sanitation of the nucleotide pool, rather than removal of oxidized lesions from DNA (Cooke *et al.*, 2008; Cooke *et al.*, 2009). In this current study, the genoprotective effects of green tea on whole body oxidative stress and oxidation-induced DNA damage were investigated using urine 8-oxodG in combination with the Fpg-assisted comet assay.

In order to fully understand the results from the current study, it is necessary to clarify the origin of 8-oxodG and another product, 8-oxoGua. 8-oxodG is an oxidatively modified product formed by hydroxyl radical reaction on a) the C8 position of the 2'-deoxyguanine base (DNA level oxidation of guanine), or b) by

oxidation of deoxyguanosine triphosphate (dGTP) or diphosphate (dGDP) (nucleotide level oxidation). Due to the highly mutagenic activity of 8-oxodG, various pathways exist to remove 8-oxodG from the nucleotide pool and from DNA levels. Sanitization of the nucleotide pool is done by 8-oxo-dGTpase (hMTH1) and 5'- nucleotidase (Haghdoost *et al.*, 2006; Cooke *et al.*, 2009). In order to prevent 8-oxodGTP in the nucleotide pool being incorporated into DNA, hMTH1 recognizes 8-oxo-dGTP and degrades it to 8-oxo-dGMP, then 5'- nucleotidase further degrades 8-oxo-dGMP to 8-oxodG, and this finally appears in urine (Cooke & Evans, 2007; Cooke *et al.*, 2008). It is possible that 8-oxodG may also be from nucleotide excision repair (NER) and nucleotide incision repair (NIR) pathways. However, their contribution to urine 8-oxodG is still controversial (Evans *et al.*, 2004; Weimann *et al.*, 2004). The action of hOGG1 and hOGG2 in the BER pathway removes 2'-deoxyguanine base lesions in DNA via cleavage of the glycosidic bond (the link between base and sugar moiety), and this releases 8-oxoGua, not 8-oxodG (Lunec *et al.*, 2002; Rozalski *et al.*, 2005). Therefore, while the origins of these in urine are still not completely identified (Cooke *et al.*, 2008), 8-oxoGua is thought to be from DNA repair, while 8-oxodG is probably from the nucleotide pool.

There are very few studies that have investigated urine 8-oxodG and to our knowledge none have investigated effects of green tea on urine 8-oxoGua. In the three previously published studies on effects of green tea on urine 8-oxodG, conflicting results were found (Young *et al.*, 2002; Hakim *et al.*, 2003; Luo *et al.*, 2006). In a controlled study of green tea extract incorporated in meat patties and

given for 3 weeks to a group of 16 smokers and non-smokers, no tea-associated changes in urine 8-oxodG (measured by HPLC with HPLC-ECD) were seen (Young *et al.*, 2002). Hakim *et al.* (2003), studied heavy smokers in a controlled 4-month intervention trial. Urine 8-oxodG (measured by an immunoassay method, which is not specific and gives much higher values than HPLC-ECD or LC-MS/MS methods) was reported to have decreased significantly after 4 months' supplementation in 42 subjects given green (4 cups/day). No significant changes were seen at months 1, 2 or 3. In a controlled study of subjects at high risk of liver cancer (Luo *et al.*, 2006), 42 subjects were given 500 mg/day green tea polyphenols in capsule form, 41 were given 1,000 mg/day and 41 subjects were given placebo. Urine 8-oxodG (measured by HPLC-ECD) was unchanged after 1 month, but was reported to be significantly lower ($P<0.01$) after 3 months' treatment with 500mg/day and with 1,000 mg/day green tea polyphenols.

In this current study, LC-MS/MS was used to measure urine 8-oxodG quantifier ion with m/z = 168. This is the preferred method, owing to its high sensitivity and specificity (Cooke *et al.*, 2008; Lee *et al.*, 2010). Results showed no significant change of urine 8-oxodG after 4 weeks' consumption of either Loongjin green tea or Screw-shaped green tea (300ml/day of 1% w/v tea). The beneficial effects of green tea may be through an accumulation of antioxidant or other components, and the dosage and duration of supplementation could be critical factors. It is noted that significantly decreased urine 8-oxodG was found in studies (Hakim *et al.*, 2004; Luo *et al.*, 2006) with higher dosage (4 cups of tea per day or

the equivalent in green tea polyphenols) and longer duration (up to 4 months) than this and the previous study that showed no effect. This current study used 2 cups/day for 4 weeks, and a green tea extract equivalent to 1-2 cups/day was given for 3 weeks in the study (Young *et al.*, 2002). In addition, beneficial effects were found in subjects under high oxidative stress (heavy smokers and hepatitis B carriers). Effects might be difficult to detect in healthy subjects, such as our volunteers. It is noted also that intra-individual (biological) variation in urine 8-oxodG excretion is wide, even when it is standardized to creatinine concentration (Lee *et al.*, 2010). Therefore, a larger study of longer duration may be needed to detect effects of dietary changes, including green tea supplementation, on urine 8-oxodG in healthy subjects.

The strengths and novelty of this current study were the design, which was a placebo-controlled cross-over trial, the use of two teas (which were of similar antioxidant content), the very sensitive and specific LC-MS/MS method used to measure the 8-oxodG, and the parallel testing of oxidation-induced basal DNA damage in lymphocytes, assessed using the Fpg-assisted comet assay. No previously published study has used both these biomarkers in the study of green tea effects. The major limitation of the study is the sample size. The numbers recruited (n=18) for the study are not particularly small for a controlled cross-over study. However, the number of complete data sets was unfortunately only 14. Post-hoc power calculation showed that this sample size is able to detect a true difference of 0.48 nmol 8-oxodG/mmol creatinine with 80% power at $p<0.05$. Given the results, which

showed normal healthy subjects in our local population have an average urine 8-oxodG of ~1.3 nmol/mmol creatinine, a change in 0.48 represents a large effect (~37%). As noted above, a larger study is needed to be able to detect smaller effects that might be induced by tea, or other dietary agents, in healthy subjects. A rewarding approach might be to investigate effects on subjects under increased oxidative stress, such as Type 2 diabetes subjects. Another limitation is that we did not measure polyphenols in plasma or urine, and so have no direct evidence of absorption of tea antioxidants.

Although effects of green tea supplementation on urine 8-oxodG were the main focus of this part of my study, it is noted that there was a significant decrease seen in basal oxidation-induced damage after each tea treatment. The decrease was fairly large (~30%) and was very similar with both types of tea. Basal damage was assessed using the Fpg-assisted comet assay. Fpg is the homologue of hOGG1 and is a base excision repair enzyme that recognizes (predominantly) 8-oxoGua within DNA (Cooke *et al.*, 2003; Smith *et al.*, 2006). As noted earlier, urine 8-oxodG is probably not from repair of oxidized lesions in DNA but from removal of oxidized guanosines in the nucleotide pool. Interestingly, in this current study no significant correlation was found between urine 8-oxodG and Fpg comet assay results. These results must be regarded as preliminary because of the small numbers in this study and the lack of any published data on inter-relationship between these, but the lack of correlation might reflect the fact that these biomarkers reflect different things. To confirm this, it would be valuable to look at the relationship between urine 8-

oxoGua and lymphocytic Fpg labile sites, and between 8-oxodG and 8-oxoGua in urine.

The question remains, why was a protective effect seen with tea in regard to basal oxidation-induced DNA damage but no effect seen on urine 8-oxodG, a putative biomarker of whole body oxidative stress? The genoprotective effects of green tea may be due to direct antioxidant effects of tea components. In our previous study it was shown that the total antioxidant content (FRAP value) of human plasma after a single dose of green tea increased by about 4 % within 40 mins (Benzie *et al.*, 1999). Even though the components of green tea that caused this increase were not identified, this may indicate some green tea antioxidant compounds are absorbed by the human body and may help lower oxidative modification of DNA. However, unless the components are somehow targeted to DNA protection it is difficult to explain why they would not also protect the nucleotide pool. There is no previous study that has investigated the effect of green tea supplementation on basal oxidation-induced DNA. There is only one published study that has used the comet assay in a green tea supplementation study (Erba *et al.,*2005), in which it was reported that lymphocytes from 10 subjects who took green tea (2 cups/day for 6 weeks) had higher resistance to Fe^{2+}- induced oxidant challenge ($P<0.05$). Some studies have looked at effects of green tea supplementation on antioxidant defences and other biomarkers of oxidative stress. However, results are conflicting (Young *et al.*, 2002; Erba *et al.*, 2005), and there is no consensus on the effect of green tea supplementation oxidative stress biomarkers.

An alternative mechanism of genoprotection could be through increased DNA repair. Future study looking at hOGG1 activity, for example using the cell extract version of the comet assay (Collins *et al.*, 2001b) and excretion of 8-oxoGua (which comes from BER) would be valuable to investigate this mechanism. Another possible mechanism is via the 'pro-oxidant' activity of antioxidants (Benzie & Wachtel-Galor, 2010). Green tea polyphenols generate hydrogen peroxide *in vitro* and can damage DNA (Suh *et al.,* 2010; Yang *et al.*, 2000). It is reported that catechins can activate a redox sensitive gene promoter region the Antioxidant Response Element (ARE) (Chen *et al.*, 2000). It is not known if either of these effects of tea antioxidants occurs *in vivo*. However, since the ARE upregulates various cytoprotective processes, including antioxidant defences and DNA repair (Lau *et al.*, 2008; Li *et al.*, 2008), activation of the ARE by tea antioxidants could result in lower DNA damage by better defence, enhanced repair, or a combination of the two as a result of an indirect effect of tea antioxidants .

In conclusion, results of this part of the study presents the first evidence that green tea has a significant genoprotective effect, which supports it use in health promotion. However, green tea supplementation had no apparent effect on a biomarker of whole body oxidative stress (urine 8-oxodG). No significant correlation was found between urine 8-oxodG and basal oxidation-induced DNA damage in lymphocytes. This may be due to the small sample size, or the genoprotective effects of green teas may not be due to simple, direct "global" antioxidant action. A larger trial is needed to detect small changes in the highly

variable urine 8-oxodG. Furthermore, it would be valuable to look at the relationships between urine 8-oxodG, urine 8-oxoGua, oxidation-induced DNA damage, and hOGG1 activity.

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(with Han KC, Benzie IFF)

Chapter 6

EFFECT OF BILBERRY ON DNA REPAIR IN TYPE 2 DIABETES MELLITUS PATIENTS

Introduction

DM can be described as a metabolic disease characterized by hyperglycaemia. The hyperglycaemia is caused by defects in insulin secretion or insulin action, or both (ADA, 2010). Diabetes can be classified into Type 1 DM (insulin dependent diabetes mellitus) and Type 2 DM (non-insulin dependent diabetes mellitus). Type 1 DM is caused by lack of insulin production due to B cell destruction by an autoimmune process (Daneman, 2006). Type 2 DM is caused by insulin resistance and insulin deficiency, and is strongly related to ageing and central obesity (Evans *et al.*, 2002). Around 97% of DM patients have Type 2 DM (Adeghate *et al.*, 2006). Since western lifestyle and obesity become more prevalent in China, the number of people suffering from Type 2 DM in China is increasing. It is estimated that at least 300 million people will have Type 2 DM in 2025, and China will account for 37.6 million cases (Narayan *et al.*, 2000). Increased oxidative stress is found in DM, and disturbed glucose/insulin homeostasis is believed to be the cause, though the exact relationship is not yet clear (Ma *et al.*, 2005; Choi *et al.*, 2008). Prolonged increases in oxidative stress may contribute to the development of serious diabetic complications such as cardiovascular disease, nephropathy, neuropathy, and retinopathy (Narayan *et al.*, 2000; Rahimi *et al.*, 2005).

Epidemiological and experimental studies show cancer risk is increased in Type 2 DM patients, though the mechanism behind this is still not fully understood (Wolf *et al.*, 2005). Increased levels of insulin or insulin-like growth factor causing increased cell division may be the cause (Giovannucci, 2001). However, for cancer to develop DNA damage must be sustained and go unrepaired. DNA damage, assessed using the comet assay, is significantly increased in Type 1 and Type 2 DM patients (Collins *et al.*, 1998; Choi *et al.*, 2005). This may be due to increased ROS production in hyperglycaemic states, or to depleted antioxidant defence (Choi *et al.*, 2008). Interestingly, DNA damage was shown to correlate significantly with glycosylated haemoglobin (HbA_{1C}) (a biomarker of 'average' plasma glucose in the previous 3 months) and fasting plasma glucose, and to correlate independently, significantly, and inversely with plasma ascorbic acid, a major water soluble antioxidant (Chung *et al.*, 2001; Choi *et al.*, 2005). In addition, less repair in lymphocytes from Type 2 DM subjects was seen (using the comet assay) after cells were exposed to oxidant challenge with H_2O_2 (Blasiak *et al.*, 2004). Increased oxidative attack, decreased antioxidant defence, and decreased DNA repair would all contribute to an increase in DNA damage and, thereby, to cell dysfunction and to increased risk of cancer.

Ideally, in a dividing cell, all DNA damage is repaired before replication (Christmann *et al.*, 2003). If DNA damage escapes from the repair system, the irreversible DNA change in daughter cells may initiate carcinogenesis (Gorbunova *et al.*, 2007). The importance of DNA repair can be demonstrated in xeroderma

pigmentosum (XP) patients, in whom the nucleotide excision repair (NER) pathway is deficient and who suffer high rates of skin cancer (Rass & Reichrath, 2008), and also in those with the autosomal recessive syndrome of adenomatous colorectal polyposis, in whom the BER pathway is deficient and high rates of colorectal cancer are seen (Cheadle & Sampson, 2003). Even in normal subjects, improved DNA repair could be expected to be beneficial, and in those under increased oxidative stress and whose baseline level of DNA damage is high, such as Type 2 DM patients, increased DNA repair could have a highly positive impact on long term health. A therapy or agent that could increase antioxidant defence, improve glycaemic control, and increase DNA repair would be very valuable in management of diabetes as it offers a 'three-pronged' benefit.

Bilberry (*Vaccinium Myrtillus L*) is a small, purplish-black berry that is rich in flavonoids named anthocyanins (Cignarella *et al.*, 1996; Canter & Ernst, 2004). Many other berries, including cranberry and strawberry, are also rich in these colourful antioxidants (Zafra-Stone *et al.*, 2007). Anthocyanins have various reported therapeutic activities including antioxidant, anticancer, anti-inflammatory and cardioprotective effects (Prior & Wu, 2006; Zafra-Stone *et al.*, 2007). Bilberry is reported also to have hypoglycaemic effects (Matsui *et al.*, 2006). Anthocyanins have powerful scavenging antioxidant activities (Kowalczyk *et al.*, 2003). Pedersen *et al.* showed that the plasma antioxidant capacity (as the FRAP value) of nine healthy women significantly increased after a single dose of cranberry juice (500 ml)

and peaked after 60 mins. This may indicate some anthocyanins are absorbed (Pedersen *et al.*, 2000).

Anthocyanins show DNA protective effects in both *in vitro* (cell culture) and animal studies. In cell culture, purified anthocyanins protected against H_2O_2 induced DNA strand breaks in human colon cells (Pool-Zobel *et al.*, 1999), and against *tert*-butyl-hydroperoxide induced DNA strand breaks in rat hepatoma and smooth muscle cells (Lazze *et al.*, 2003). However, *in vitro* results need to be confirmed *in vivo*. Spormann *et al*. showed that after 4 weeks' anthocyanin-rich fruit juice (200 ml/day) consumption, the specific sites detected by the Fpg-assisted comet assay were significantly lowered in 21 haemodialysis patients (Spormann *et al.*, 2008). However, a controlled study in which 20 healthy women were given 750 ml/day anthocyanin-rich cranberry juice or placebo for 2 weeks showed no cranberry-associated changes in any biomarkers of antioxidant status, and baseline and H_2O_2-induced DNA damage (Duthie *et al.*, 2006). Therefore, the effect of anthocyanin-rich fruits or juices or extract on DNA damage in humans is not yet confirmed. Furthermore, most of the published studies have used purified anthocyanins or mixed fruit juices, and no study to date has looked at effects of anthocyanin-rich berries, such as bilberry, on DNA repair. This was the main focus of this part of the study, in which we studied effects of anthocyanin-rich bilberry extract on hOGG1 activity (the first step in BER) in a controlled human supplementation trial of cross-over design. The subjects were Type 2 DM patients. Because of earlier reports that DNA damage is related to glycaemic control and ascorbic acid levels (Choi *et al.*,

2005), it was of interest to investigate if DNA repair (hOGG1 activity) was also related to these.

Materials And Methods

The method used to investigate DNA repair effects was the cell extract version of the comet assay with damaged substrate cells. This method was described in detail in Chapter 3. To recap briefly, substrate HeLa cells were damaged in a controlled and "standardized" manner by the action of a photosensitizer (Ro 19-8022) and light, and then treated with a cell (lymphocyte) extract that contained hOGG1. The action of hOGG1 creates strand breaks at oxidation-induced lesions (predominantly 8-oxoGua), and these are measured by the comet assay. The greater the hOGG1 activity, the more damage will be revealed, i.e., higher comet assay damage scores in substrate cell after cell extract treatment = higher hOGG1 activity in cell extract. In this part of the study, the cell extracts were prepared (described in Chapter 3) from lymphocytes harvested from fasting venous blood of Type 2 DM subjects before and after supplementation with bilberry extract and placebo (supplementation details are given below). For the other biomarkers of interest (HbA_{1C} , fasting plasma glucose and fasting plasma ascorbic acid) samples were measured by another member of our research group, and data were retrieved by this investigator. The commercial hexokinase spectrophotometric kit method for plasma glucose was from Roche Diagnostic Systems (Basel, Switzerland). Cfas from Roche Diagnostic Systems was used for calibration, and Precinorm U and Precipath U from Roche Diagnostic Systems were used as controls. The commercial kit for

HbA_{1C} was from BioSystems (Barcelona, Spain) and used chromatographic separation. The FRASC assay for ascorbic acid is a modified version of the FRAP assay (Benzie & Strain, 1999; Chung *et al.*, 2001). In this assay, ascorbic acid is selectively destroyed in the sample by the addition of ascorbate oxidase to one of a pair of sample aliquots. The difference in absorbance between the paired samples is due to ascorbic acid. This method has been validated against an HPLC reference method for ascorbic acid (Chung *et al.*, 2001).

Supplementation Scheme

This was a double-blinded, placebo-controlled cross-over intervention trial with 20 Type 2 DM subjects (9 male, 11 female), aged between 31 and 70 years. Subjects were recruited from the diabetic and general medical clinic in the Prince of Wales Hospital, Shatin, Hong Kong. Written informed consent was obtained from each subject before the study began. This study was approved by the Joint Chinese University of Hong Kong (CUHK) and New Territories East Cluster (NTEC) Clinical Research Ethics Committee (CREC) and by The Human Subjects Ethics Subcommittee of the Hong Kong Polytechnic University (Appendix 3). Subjects were under medical supervision within the study period. Exclusion criteria included smoking, regular intake of antioxidant or vitamin supplementation or Chinese medicine, presence of diabetic complications, previous history of heart disease or stroke, chronic disease (apart from diabetes), body mass index (BMI) below 23 kg/m^2. Subjects were allocated, on non-selective basis, to receive 4 weeks' supplementation with (i) bilberry capsules (2 capsules in morning and 2 capsules in

evening), or (ii) placebo capsules (2 capsules in morning and 2 capsules in evening). Half started with placebo and half with bilberry. Treatments were coded, and subjects and this investigator did not know which treatment was given each time. Codes were broken after data were collected. After 6 weeks' washout period, the procedure was repeated with subjects crossed-over onto the other treatment. Commercial bilberry and placebo capsules used were a kind gift from PolyU Technology & Consultancy Co., Ltd (PTeC) and were from Guilin Layn Natural Ingredients Corp. Each capsule contained 0.2 g bilberry powder (or starch for placebo). The Certificate of Analysis of the bilberry extract in the capsules is given in Appendix 4. The anthocyanins content was ~25.6% in each capsule. Therefore the daily dose of anthocyanins in this study was ~205 mg. Compliance was assessed by inquiry and by counting returned capsules at the end of each intervention: return of <85% was regarded as satisfactory compliance (i.e., return of <15% of the number of capsules that should have been consumed was regarded as satisfactory compliance). Twelve ml of fasting venous blood was collected before and after each treatment, 2 ml into EDTA blood collection tube (for HbA_{1C}), 2 ml into fluoride oxalate blood collection tube (for glucose) and 8 ml into heparinised blood collection tubes (for ascorbic acid and for lymphocyte harvesting). The study protocol is outlined in Figure 6.1.

Statistical Analysis

Wilcoxon matched pairs test was used to investigate the DNA repair responses to treatment. For the correlation study, Pearson coefficient of correlation was used. $P<0.05$ was regarded as statistically significant. Prism 4.0 (Graphpad Software, San Diego, CA, USA) was used. No *a priori* power calculation was performed. Resource limitations restricted the duration and size of the trial, but 20 subjects was selected as initially feasible and this number is not regarded as small in this type of study. Further, there are no published SD data on the key variable (the index of DNA repair, measured as %DNA in comet tail of damaged HeLa cells after incubation with lymphocyte extracts) to use for power calculations. One approach is to perform a power calculation based on relative SD change as the detectable difference. Using this approach, 20 subjects in a cross-over trial can detect a true difference of ≥0.7 SD in the key variable, with 80% power and at the 5% significance level (by PS-Power and sample size calculation V3.0.4). However, it is noted that, due to missing data (non-compliant subjects (n=3) or problems in harvesting sufficient cells (in 6 samples), only 11 complete data sets were available. In *post-hoc* analysis, these 11 datasets were sufficient to detect a true difference of ≥0.9 SD of the key variable at 80% power and 5% significance level.

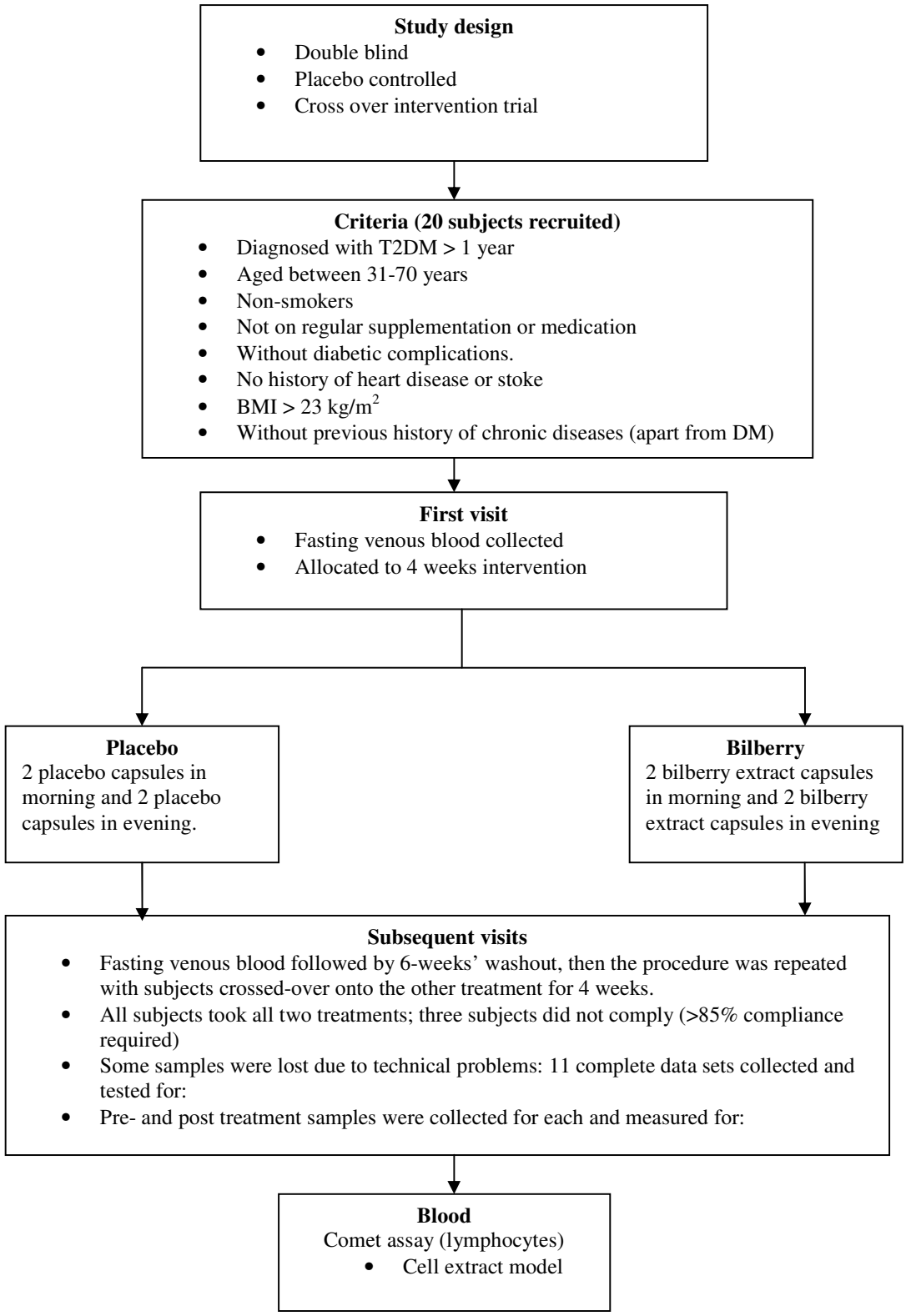

Figure 6.1. Outline of study protocol.

Results

As noted above, data from 3 subjects were excluded due to non-compliance. In addition, some samples did not yield enough cells. Therefore, only 11 complete data sets (pre and post bilberry and pre and post placebo) were available from the supplementation trial. Baseline (entry) data were available from 16 subjects for the correlation analysis.

Results of preliminary experiments are shown in Figure 6.2. These experiments were performed to confirm that: A) the background level of 8-oxoGua sites (lesion recognized by hOGG1) is low in HeLa cells; B) light alone or C) Ro 19-8022 photosensitizer alone did not induce additional 8-oxoGua; D) the photosensitizer plus light induced marked damage (as 8-oxoGua sites); in this experiment these sites were recognized and changed to strand breaks by the action of Fpg, the microbial analogue of hOGG1. It can be seen that levels of 8-oxoGua, as strand breaks, were 4-5 times higher in the Fpg-assisted comet when HeLa cells were pre-treated with Ro 19-8022 and light. This indicates Ro 19-8022 plus light can induce sufficient 8-oxoGua for detection.

Table 6.1 shows that, in HeLa cells without the induced damage from photosensitizer and light, background DNA damage is low, even in the presence of the cell extract. Table 6.2 shows that treatment of damaged substrate cells with the hOGG1-containing extracts revealed the strand breaks caused by the enzyme as the first stage of repair. However, results showed no significant effect of bilberry

treatment on the amount of damage revealed, i.e., no change in hOGG1 activity with bilberry was seen (Figure 6.3). The pre and post results of the 11 subjects from whom complete data sets were available are shown in Figure. 6.4 for placebo and 6.5 for bilberry.

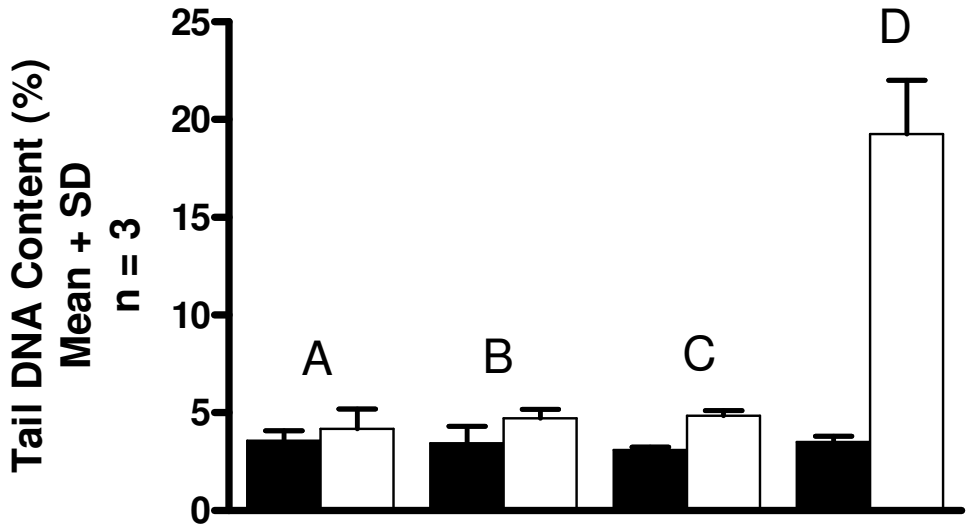

Figure 6.2. The %DNA in comet tail in HeLa cells (Solid bars represent cell with buffer treatment (no enzyme); open bars represent cells treated with Fpg enzyme: (A) no Ro 19-8022 and no light; (B) no Ro 19-8022 but with light exposure; (C) with Ro 19-8022 but no light; (D) with Ro 19-8022 and light exposure. Results are mean+SD of two gels/treatment with 50 cells scored per gel, with triplicate experiments run.

Table 6.1. Results of %DNA in comet tail in undamaged HeLa (substrate) cells treated with cell extracts from samples collected pre- and post- 4 weeks' placebo or bilberry supplementation (n=11)

	Placebo			Bilberry		
	Undamaged substrate cells			Undamaged substrate cells		
	Pre-	Post-	Change	Pre-	Post-	Change
Range	1.85 to 3.68	2.68 to 4.04	-0.64 to 2.02	2.03 to 4.16	2.72 to 4.00	-0.80 to 1.62
Median	3.28	3.63	0.37	3.29	3.58	0.01
Mean (SD)	3.08 (0.57)	3.54 (0.44)	0.45 (0.78)	3.36 (0.65)	3.49 (0.40)	0.13 (0.77)
95% range of values	2.70 to 3.46	3.24 to 3.84	-0.07 to 0.98	2.92 to 3.80	3.22 to 3.76	-0.39 to 0.64

Table 6.2. Results of %DNA in comet tail in damaged HeLa (substrate) cells treated with cell extracts from samples collected pre- and post- 4 weeks' placebo or bilberry supplementation (n=11).

	Placebo			Bilberry		
	Damaged substrate cells			Damaged substrate cells		
	Pre-	Post-	Change	Pre-	Post-	Change
Range	9.49 to 15.75	9.98 to 13.47	-3.82 to 3.56	11.16 to 13.16	9.79 to 15.35	-1.93 to 2.96
Median	11.54	11.99	0.07	12.15	11.98	-0.19
Mean (SD)	11.77 (1.70)	12.05 (1.0)	0.28 (2.16)	12.03 (0.69)	12.05 (1.58)	0.02 (1.33)
95% range of values	10.62 to 12.91	11.37 to 12.72	-1.17 to 1.73	11.57 to 12.49	10.98 to 13.11	-0.87 to 0.91

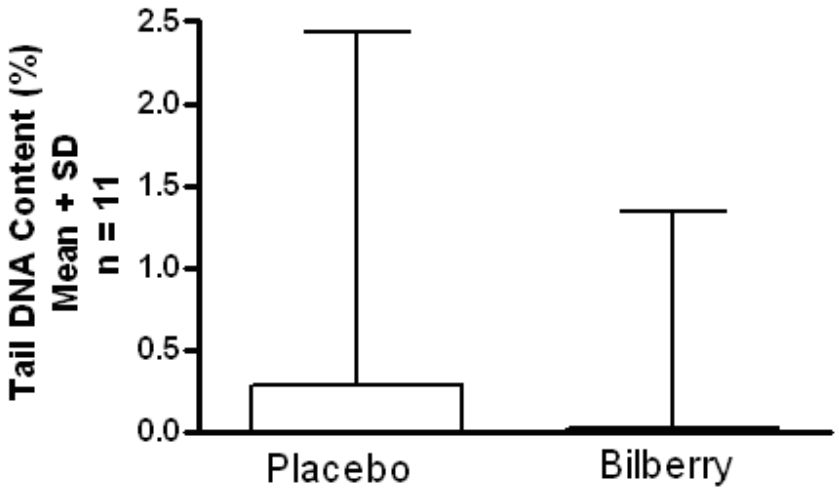

Figure 6.3 Change from baseline in tail DNA (%) in damaged HeLa cells incubated with lymphocyte extracts after 4 weeks' supplementation with placebo or bilberry. Results are mean + SD (post-pre difference) in 11 subjects.

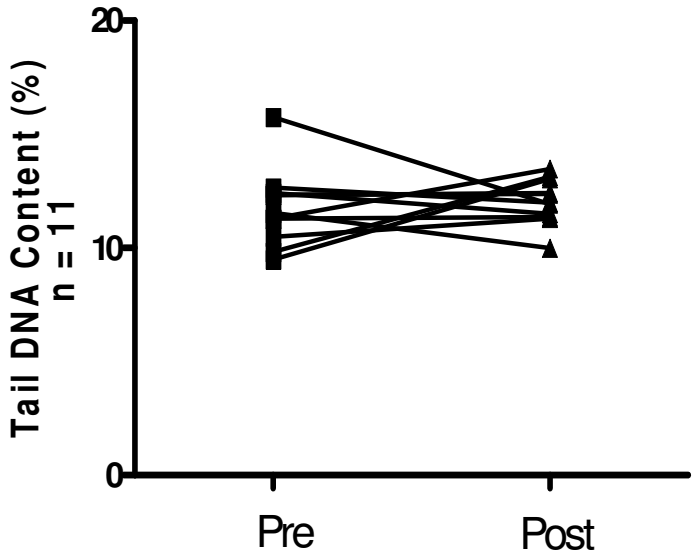

Figure 6.4 Individual results of showing changes in DNA (%) in damaged HeLa cells incubated with lymphocyte extracts before and after 4 weeks' supplementation with placebo.

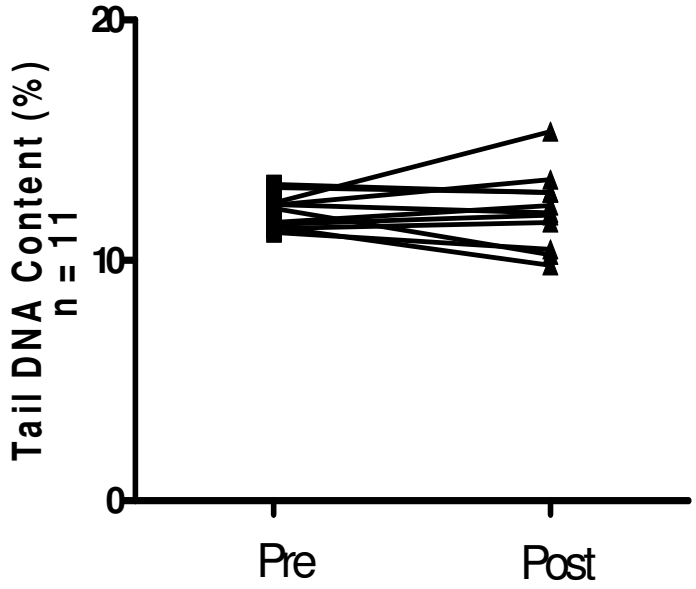

Figure 6.5 Individual results showing changes in DNA (%) in damaged HeLa cells incubated with lymphocyte extracts before and after 4 weeks' supplementation with bilberry.

With reference to possible inter-relationship between ascorbic acid or glycaemic control and hOGG1 activity, there were no significant correlations found between hOGG1 activity (using the %DNA in comet tail of damaged HeLa substrate cells treated with cell extracts as the biomarker of hOGG1 activity) with fasting plasma ascorbic, fasting plasma glucose or HbA_{1C} (Figures 6.6, 6.7. and 6.8).

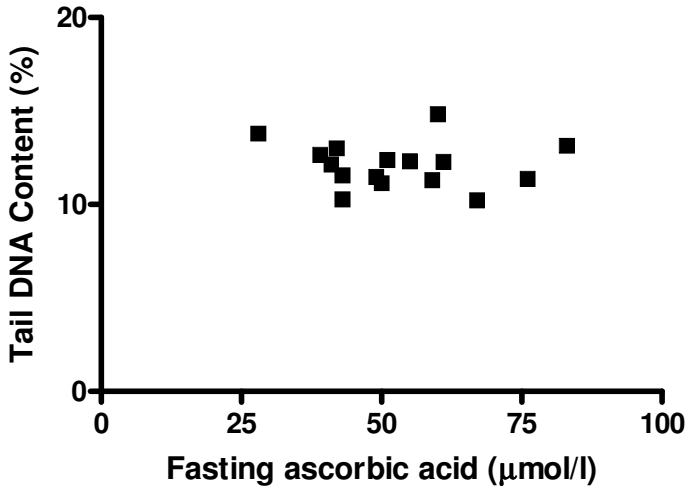

Figure 6.6. No correlation seen between fasting plasma ascorbic acid and DNA repair (as % DNA in tail of damaged HeLa cells after treatment with lymphocyte extracts obtained from samples collected at entry; Pearson's r = -0.11, P = 0.7; n = 16).

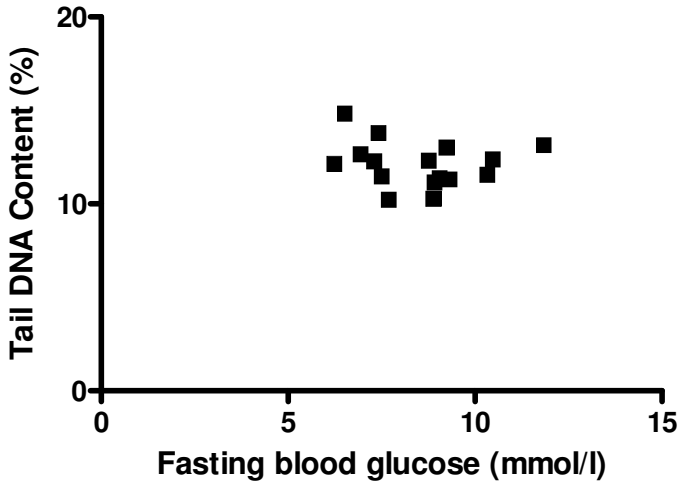

Figure 6.7. No correlation seen between fasting blood glucose and DNA repair (as % DNA in tail of damaged HeLa cells after treatment with lymphocyte extracts obtained from samples collected at entry; Pearson's r = -0.16, P = 0.54; n = 16).

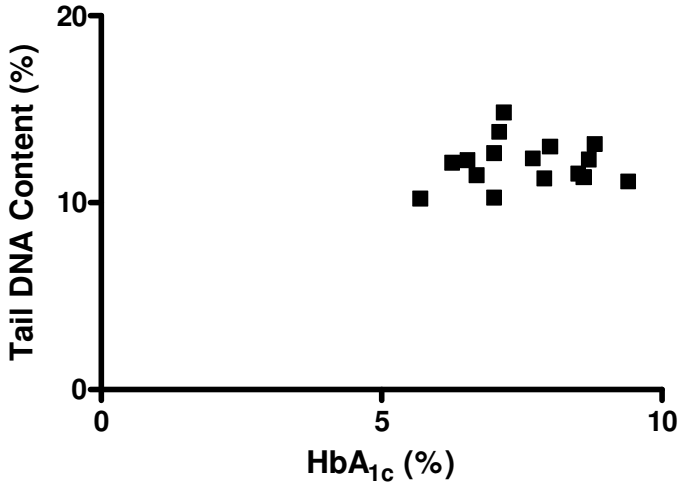

Figure 6.8. No correlation seen between HbA_{1c} and DNA repair (as % DNA in tail of damaged HeLa cells after treatment with lymphocyte extracts obtained from samples collected at entry; Pearson's r = -0.07, P = 0.79; n = 16).

Discussion

Type 2 DM is a metabolic disease characterized by impaired blood glucose control, and usually accompanied by long term microvascular (retinopathy, nephropathy and neuropathy) and/or macrovascular (cardiovascular disease and stroke) complications (Choi *et al.*, 2008; ADA, 2010). These complications are associated with poor glycaemic control and increased oxidative stress (Ohta, 2003; Brownlee, 2005; Khan *et al.*, 2006). DNA damage is increased in Type 2 DM, and this is likely due to the combination of increased oxidative stress, antioxidant depletion, hyperglycaemia, and, possibly, decreased DNA repair (Collins *et al.*, 1998; Dincer *et al.*, 2002; Blasiak *et al.*, 2004; Choi *et al.*, 2005; Choi *et al.*, 2008; Lodovici *et al.*, 2008; Sliwinska *et al.*, 2008). DNA repair is a crucial gate keeper against carcinogenesis and senescence, and is vital for overall cellular well being, especially in cases of increased DNA attack, such as DM.

Bilberry is an anthocyanin-rich berry that has been reported to have antioxidant and hypoglycaemic effects (Cignarella *et al.*, 1996; Zafra-Stone *et al.*, 2007), and offers potential health benefits for DM patients. There is no previous study of the effect of bilberry on human DNA damage or repair. There are many studies of dietary antioxidants, including anthocyanins, on Fpg labile sites and other markers of DNA damage (reviewed by Møller and Loft, 2006). Agents tested have included anthocyanin-rich juices, pure berry juices, and anthocyanin extracts (Møller & Loft, 2006). Results are inconclusive. DNA lesions in lymphocytes (detected by the Fpg-assisted comet assay) were decreased significantly after 4

weeks' supplementation of 18 healthy subjects (Weisel *et al.*, 2006) and, in a sister study (Spormann *et al.*, 2008), after 4 weeks supplementation of 21 haemodialysis subjects, both studies using supplementation with anthocyanin-rich mixed berry (but not bilberry) fruit juice (though it is noted that the juices used contained vitamin C). In contrast, Møller *et al.,* (2004) reported no decrease in Fpg labile sites in lymphocytes in 20 healthy subjects who took a drink rich in anthocyanins (supplying ~365mg/day, and containing no vitamin C) for 3 weeks. Interestingly, they reported an increase in Fpg labile sites in 18 subjects who took whole blackcurrant juice containing a similar amount (397 mg/day) of anthocyanins but which supplied also 140 mg/day vitamin C. The authors concluded that high intake of antioxidants may only have protective effects in those with increased oxidative stress (Møller *et al.*, 2004). In a study using supplementation with commercial cranberry juice (750 ml/day for 2 weeks), Duthie *et al.* (2006) reported no effect on lymphocytic Fpg labile sites of 20 healthy subjects. However, it is noted that the juice was very low in anthocyanins (2.80 mg/l), and no anthocyanins were detectable in the plasma of the subjects taking the juice (Duthie *et al.*, 2006). However, the juice was high in vitamin C (897 mg/l) and total phenols. Therefore, the effect of anthocyanins supplementation on oxidation-induced DNA damage is not clear.

The level of oxidatively modified DNA within cells is the dynamic equilibrium point between the rate of DNA damage and the rate of repair. In BER the first step is hOGG1 action (Collins *et al.*, 2008). Increased DNA repair could be

responsible for some of the positive effect of anthocyanins on basal levels of damage reported previously, although in both cases the authors attributed the findings to the polyphenol content on antioxidant defence of DNA, rather than to repair enhancement (Weisel *et al.*, 2006; Spormann *et al.*, 2008). This part of the current study focused on the effect of a bilberry extract on DNA repair in a susceptible group of people, i.e. Type 2 DM subjects. People with Type 2 DM are under increased oxidative stress (Choi *et al.*, 2008; Ma *et al.*, 2008) and their DNA repair has been reported to be decreased (Blasiak *et al.*, 2004; Sliwinska *et al.*, 2008). In this current study, hOGG1 action was assessed using a cell extract version of the comet assay before and after 4 weeks' supplementation with the bilberry extract that supplied 205 mg/day of anthocyanins. The daily intake of anthocyanins is wide, can range from a few mg to several hundreds mg/person/day (Hou, 2003), so the dose used is dietary relevant but represents an approximate doubling of usual intake. In addition, since plasma ascorbic acid and glucose control are related to basal oxidation-induced DNA damage levels (Collins *et al.*, 1998; Choi *et al.*, 2005), it was of interest to see if these factors related to DNA repair of oxidation induced DNA damage. No previous study has investigated these relationships. The results showed no significant effect of bilberry supplementation on hOGG1 activity in lymphocytes of the Type 2 DM patients tested. Furthermore, no significant correlations were seen between hOGG1 activity and glycaemic control or plasma ascorbic acid.

The evidence that DNA repair is decreased in Type 2 DM patients comes from two studies by a Polish group (Blasiak *et al.*, 2004; Sliwinska *et al.*, 2008).

Both studies used the comet assay to measure DNA repair immediately and 2 hrs after H_2O_2 challenge. In Blasiak's study (2004), 52 Type 2 DM patients and 55 control subjects were recruited, and lymphocytes were challenged with 10 μmol/l H_2O_2 on ice for 5 mins. The study reported that challenged cells from Type 2 DM patients had 48% damage remaining after 2 hrs, whereas cells from the control group had only 36% remaining ($P<0.05$) (Blasiak *et al.*, 2004). In Sliwinska's study (2008), lymphocytes from 30 Type 2 DM patients and 30 control subjects were challenged with 10 μmol/l H_2O_2 on ice for 10 mins. After 2 hrs repair time, challenged cells from Type 2 DM patients showed 20% damage remaining, compared to 10% in cells from normal subjects ($P<0.001$) (Sliwinska *et al.*, 2008). Together, these two studies suggest that there is ~10% less repair in Type 2 DM patients. However, it is not clear if DNA repair was simply slower or if it was less effective, and the part of the BER pathway affected was not revealed because the model used in these studies assesses overall repair of strand breaks. The cell extract model used in this study assesses the first step in the BER pathway, hOGG1 activity, specifically. There are no previously published studies that have used the cell extract model to assess DNA repair in Type 2 DM subjects. There is one study (Tyrberg *et al.*, 2002) that investigated hOGG1 protein expression in pancreatic tissue of normal and Type 2 DM subjects (n=5 for each). Using immunofluorescence staining, the authors found significantly more hOGG1 in the diabetic pancreatic tissue, and concluded that hOGG1 is upregulated in pancreatic islet tissue in Type 2 DM (Tyrberg *et al.*, 2002). The authors also suggested that this effect was linked to increased oxidative stress in association with hyperglycaemia

(Tyrberg *et al.*, 2002). Increase in hOGG1 protein expression has been reported by Duthie *et al.* in association with increased DNA damage and folate deficiency (Duthie *et al.,* 2010). However, an increase in hOGG1 in diabetes would be expected to increase repair, and is in contrast to the findings of the Polish studies mentioned above and to results of an animal study that investigated effect of hyperglycaemia on hOGG1 expression in renal tissue of rats with strepotozotocin-induced DM (Simone *et al.*, 2008), in which expression of hOOG1 was reported to be markedly lower in diabetic rat renal cortex compared to that of control animals. The study also looked at effects of incubation in high glucose (25 mmol/l for up to 60 mins) on hOGG1 protein expression in primary cultures of renal cortical tissues and murine proximal tubular tissue. In both types of renal tissues exposed *in vitro* to high glucose, hOGG1 protein was much lower after 60 mins' glucose exposure. The authors concluded that hOGG1 expression was downregulated via the PI 3-kinase/Akt/tuberin pathway (Simone *et al.*, 2008). Hyperglycaemia increases ROS production (Choi *et al.*, 2008), including H_2O_2, and this ROS can directly activate Akt which in turn enhances tuberin phosphorylation and finally down-regulates hOGG1 (Simone *et al.*, 2008). Mild oxidative stress might increase hOGG1 through its effects on the Antioxidant Response Element (ARE) (Pi *et al.*, 2003; Mo *et al.*, 2006; Benzie & Wachtel-Galor, 2010). However, while levels at 60 mins were low, closer inspection of the data shows increased hOGG1 in these rat renal cells at 15 and 30 mins in the high glucose environment (Simone *et al.*, 2008). The overall effect of these hOGG1 changes on DNA repair is not clear and was not tested, and whether or not BER is affected in Type 2 DM remains to be confirmed. It is

possible that increased oxidative stress could increase or decrease hOGG1, and BER, depending upon the size or duration of the stress and degree of hyperglycaemia or level of current DNA damage. In this study, we did not assess oxidative stress, a baseline level of DNA damage, but we saw no correlation between hOGG1 activity and glucose level or HbA_{1C}, or a measure of antioxidant defence, the plasma ascorbic acid concentration.

This study showed that supplementation with an anthocyanin-rich bilberry extract did not increase hOGG1 activity in Type 2 DM subjects. The genoprotective effect of anthocyanins reported in previous studies (Weisel *et al.*, 2006; Spormann *et al.*, 2008) may be due to the potent antioxidant power of these phenolic compounds (Pool-Zobel *et al.*, 1999; Weisel *et al.*, 2006; Zafra-Stone *et al.*, 2007) and, possibly, the ability of anthocyanins to stabilize DNA. Through formation of a complex with DNA, anthocyanins are reported to protect DNA against oxidative damage (Sarma & Sharma, 1999).

This current study had several limitations. Unfortunately there were only 11 complete supplementation datasets, even though 20 people were recruited. As noted above, the literature on genoprotective effects of anthocyanins is conflicting. In this study we did not investigate effects of anthocyanin-rich bilberry on basal Fpg labile sites because of sample limitations and other resource restrictions. However, in further study it would be useful to look again at protection (Fpg labile sites) especially in subjects under increased oxidative stress, such as Type 2 DM subjects,

and if possible to look at hOGG1 expression in relation to oxidative stress and glycaemic control in human subjects. Another limitation of this study is that we did not measure plasma or urine levels of anthocyanins. This was due to resource limitations. The lack of effect on hOGG1 may be due to poor bioavailability of anthocyanins, so that even though the supplement represented a doubling of the normal dietary daily intake, plasma and cellular levels may have remained too low to have an effect. The plasma anthocyanins concentration reaches a maximum ~70 mins after oral ingestion, and the elimination half-life of plasma anthocyanins is around 130 mins (Upton, 2001). However, the absorption of anthocyanins is poor, and in a human study, plasma peak plasma concentration (Cmax) was only ~100 nmol/l after ingestion of 720 mg anthocyanins, showing low bioavailability (Upton, 2001). Still, even with low bioavailability, regular intake of a dietary agent could have some beneficial effects in the long term. These might be due to direct antioxidant action, DNA stabilization, through effects on the ARE, or to as yet unknown mechanisms.

In conclusion, results presented in this part of study demonstrate that anthocyanin-rich bilberry extract did not increase hOGG1 activity after 4 weeks supplementation in Type 2 DM patients, and hOGG1 activity did not show significant relationship with glycaemic control or fasting plasma ascorbic acid. The lack of effect may have been due to the small sample size and the dosage used and low bioavailability of anthocyanins. This can only be regarded as a preliminary study. In addition, if DNA repair in Type 2 DM patients is not low, as has been

previously reported, any effects of diet or therapy would be difficult to detect as they would be very subtle and small. The new data presented here will be useful in planning larger, longer term studies of effects on anthocyanins and bilberry on DNA because we have gathered the first (to our knowledge) data on hOGG1 activity in Type 2 DM patients using the cell extract comet assay, and these can be used for comparison with other groups, including healthy subjects, and for power calculations for follow-up studies using bilberry and other potential therapeutic agents targeting hOGG1. A longer term intervention trial with adequate power in Type 2 DM subjects with different levels of glycaemic control and oxidative stress is needed to clarify if bilberry has an effect on hOGG1 activity or on basal oxidation induced damage. Furthermore, it would be worthwhile to look at DNA protection and repair especially in relation to different levels of oxidative stress and glycaemic control.

Chapter 7

PHOTOPROTECTIVE POTENTIAL OF CORDYCEPS POLYSACCHARIDES AGAINST ULTRAVIOLET B RADIATION-INDUCED DNA DAMAGE TO HUMAN SKIN CELLS

Introduction

UV radiation is an environmental genotoxic agent with well documented adverse effects on skin (Cleaver & Crowley, 2002; Marrot & Meunier, 2008; Moan *et al.*, 2008). Solar UV radiation is composed of UVA (320-400nm), UVB (290-320 nm) and UVC (200-290 nm) radiation (Bens, 2008). Short wavelength UVC has the highest energy, and can penetrate deep into the skin and induce severe damage (Bens, 2008; Rass & Reichrath, 2008). Fortunately, UVC is almost completely absorbed by the Earth's ozone layer, and although there are concerns about ozone thinning leading to increased exposure to UVC in some parts of the world, UVC is not regarded as a major problem (Rass & Reichrath, 2008).

The UVA and UVB irradiation that reaches the skin surface is partially reflected by the stratum corneum of the epidermis, and partially absorbed by epidermal melanin (Bens, 2008). However, a significant amount of UV escapes these barriers and can induce DNA, and other, damage to skin cells, causing photoageing and increasing risk of skin cancer (Figure 7.1). UVA and UVB affect

different layers of the skin, and induce damage to DNA by different mechanisms (Rass & Reichrath, 2008). Up to 50 % of UVA reaches the dermis and can damage DNA indirectly by Type I and II photosensitization reactions. These involve ROS which cause oxidation-induced lesions, such as 8-oxodG, within DNA (Kielbassa *et al.*, 1997; Hiraku *et al.*, 2007; Cooke *et al.*, 2009). UVB is a relatively small portion (~5 %) of the UV radiation reaching the Earth's surface, and only 10-20 % of this penetrates the epidermal layer. UVB generally does not cause large amounts of oxidation-induced changes to DNA. However, it is notable because UVB is of high energy and it interacts directly and quickly with DNA in skin cells, creating characteristic types of DNA damage, the cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine-pyrimidinone photoproducts (6-4 PPs) (Kielbassa *et al.*, 1997; Cleaver & Crowley, 2002; Bens, 2008; Hakozaki *et al.*, 2008). Both types of dimer are generated at di-pyrimidine sites within the DNA strand, and can cause C → T transition mutations. The thymine-thymine dimer is the most common type of CPD, whereas the thymine-cytosine dimer is the most common type of 6-4 PP. The CPD dimer is the predominant lesion caused by UVB, takes longer to repair than other type of DNA damage, and is regarded as more mutagenic (Riou *et al.*, 2004; de Lima-Bessa *et al.*, 2008). The C → T transition mutation at hotspots in the *p53* gene is related to the most common type of skin cancer, basal cell carcinoma (Ziegler *et al.*, 1993). The relationship between UVB and melanoma is less well understood, but melanoma incidence rate in xeroderma pigmentosum (XP) patients, in whom the NER pathway, used to repair CPDs, is deficient, is increased by UVB exposure (Cleaver & Crowley, 2002). Therefore, while less UVB than UVA reaches skin,

UVB radiation specifically targets DNA, and is thus of special interest as an environmental genotoxin in relation to both melanoma and non-melanoma types of skin cancers.

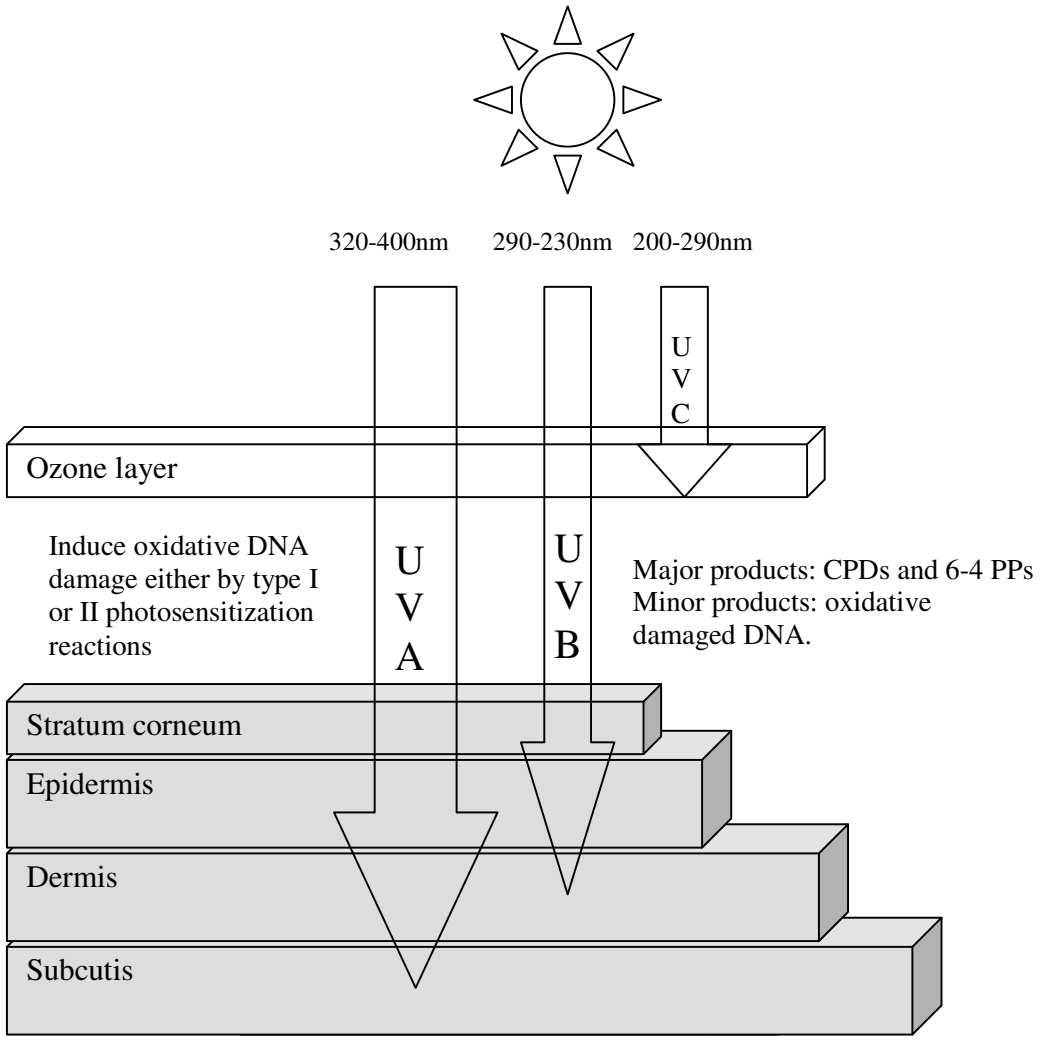

Figure 7.1. Penetration and reaction of UV radiation in human skin.

There is, generally, effective repair of UVB-induced damage (Ziegler *et al.*, 1993; Rass & Reichrath, 2008). However, protection to minimize damage is crucial, especially in situations where exposure to UVB is high, when skin pigmentation is

low, or if DNA repair is compromised. Sunscreens are commonly used to protect against the harmful effect of UV radiation by reflection, absorption and scattering of UV radiation (González *et al.*, 2008). Ideally, protection should not be limited to UV screening, but also oppose DNA damage by direct or indirect protection. Increased DNA repair would also be protective against long term effects of damage (González *et al.*, 2008).

Natural products are attracting much interest for a range of health benefits, including protection against photo-induced damage to skin. Among the best known natural products for this to date are green tea polyphenols. These have strong UV absorption characteristics and may be useful sunscreens (Wei *et al.*, 2009). Furthermore, as UV radiation (mainly UVA) creates oxidation-induced damage to skin, dietary antioxidants, such as β-carotene, α-tocopherol, ascorbic acid and green tea polyphenols have been studied for their protective effects against this (Offord *et al.*, 2002; Morley *et al.*, 2005). However, UVB induces most DNA damage due to a direct and non-oxidative process. Therefore, in the study of skin protection, it is important also to focus on the characteristic DNA lesions induced by UVB, that is, the pyrimidine dimers. It has been shown that very low doses of UVB can induce significant amounts of lesions that are recognized by the T4 endonuclease V (T4EV) without producing detectable oxidation-induced DNA lesions (Kielbassa *et al.*, 1997). Therefore, the T4EV-assisted comet assay offers a valuable tool for the investigation of UVB irradiation-induced DNA damage and protection against this by natural products.

Cordyceps sinensis (Berk.) Sacc., also known as the Chinese caterpillar fungus, is a parasitic fungus of the caterpillar of the moth *Hepialis armoricanus* (Paterson, 2008; Zhou *et al.*, 2009). This medicinal fungus has been reported to have numerous pharmacological properties, including antioxidant, anti-inflammatory and anti-tumour effects (Leung *et al.*, 2006). There is a high and increasing global demand for Cordyceps. However, natural Cordyceps, which is a combination of caterpillar body and the fungal fruiting body, is only found at high altitudes on the Himalayan Plateau (Zhou *et al.*, 2009). It is rare and cannot meet the increasing demand. Various fermentable strains of Cordyceps fungi have been developed, and Cordyceps mycelium cultures are the major sources of commercial Cordyceps products (Paterson, 2008). Studies show that the pharmacological and chemical compositions are similar between natural and cultivated Cordyceps (Li *et al.*, 2001).

Polysaccharides in Cordyceps are the putative key bioactive agents showing notable antioxidant and anti-tumour effects (Ng & Wang, 2005; Leung *et al.*, 2006; Wu *et al.*, 2007; Zhou *et al.*, 2009). There are no studies to date on potential DNA or other protective effects of Cordyceps polysaccharides against UV radiation. However, green tea polysaccharides have been reported to protect mouse skin fibroblasts against UVA and UVB radiation (Wei *et al.*, 2009). Protection was not due to UV absorption (Wei *et al.*, 2009) suggesting some cellular photoprotective action by the polysaccharides, but DNA damage was not assessed. The aim of this study was to use the T4EV-comet assay model to investigate the potential

genoprotective effects of polysaccharide-rich components of Cordyceps mycelia in a human skin fibroblast model of UVB exposure.

Materials And Methods

The method used to investigate the UVB-protective effect of Cordyceps was the T4EV-assisted comet assay. This method was described in detail in Chapter 3. To recap the model briefly, normal human fibroblasts called BJ cells were irradiated with UVB under controlled conditions and after being embedded in agarose. UVB-induced CPDs were converted to strand breaks by T4EV. The comet assay was then performed and %DNA in comet tail was measured.

To study the potential UVB-protective effect of Cordyceps polysaccharides, the BJ cells were pre-incubated in Cordyceps polysaccharide-rich extracts and washed before being irradiated with UVB. The Cs-HK-1 extracts were prepared from mycelium cultures that were well established in The Hong Kong Polytechnic University, and Cs-HK-1 extracts were shown to have antitumour effects (Leung *et al.*, 2006; Wu *et al.*, 2007).

The details of cell culture, BJ cells preparation and cytotoxicity were described in detail in Chapter 3. To recap briefly, BJ cells were incubated with defined concentration of <500EPSCM or HWECMyc (50, 100, 200 μg/ml) in complete medium for 30 mins and 24 hrs. Cells were incubated with complete medium alone as control. After incubation, cell viability was determined by the

Trypan blue exclusion test. Other aliquots of pre-treated cells were washed and embedded in agarose on microscope slides for irratidation and the T4EV comet assay testing.

Preparation Of Cordyceps Extract

Two preparations of Cs-HK-1 Cordyceps mycelia culture were used (Leung *et al.*, 2006). These were supplied as freeze dried powder by Dr. JY Wu, Department of Applied Biology & Chemical Technology, The Hong Kong Polytechnic University. One was a hot water extract of dried mycelia biomass, referred to as 'HWECMyc'. The dry mycelium powder was extracted with boiling distilled water (1 g in 10 ml water) for two hours. The solid was removed by filtration and the liquid extract was freeze-dried. The other preparation was a crude extract prepared as a 95 % v/v ethanol precipitate of the liquid medium around the cultured mycelia. The exopolysaccharides captured from the liquid medium were redissolved in Milli-Q water. Molecular weight cut-off membranes (Millipore, Millipore Corporation, USA) were used to collect extract with exopolysaccharides of molecular weight < 500kDa and this is referred to as '<500EPSCM'. Aqueous solutions of these extracts and the agarose for embedding cells were subjected to an UV absorbance scan using a spectrophotometer (DU 730, Beckman Coulter, Inc., Fullerton, CA, USA). Solutions of extracts for adding to BJ cells were made up in complete medium. Concentrations of 0, 50, 100, 200 µg/ml were used.

UVB Irradiation Of Cells

The source of UVB (302 nm) irradiation was a single-tube lamp from UVP (Upland, Cambridge, England). The intensity of the UVB setting was monitored by a digital radiometer from Newport (Irvine, CA, USA). The cover slip was removed from the set agarose gels containing the BJ cells, and slides were placed on a cold metal plate over ice at a distance of 165 cm from the UVB lamp. Cells were exposed to UVB irradiation for 10 sec. This provided a dose of UVB irradiation of 0.23 J/cm^2. This distance and time for UVB irradiation were selected on the basis of our preliminary studies, which aimed to find conditions that induced minimal overt DNA damage in BJ cells in the comet assay without the use of T4EV treatment, but that clearly induced CPDs, as revealed by the enzyme-assisted comet assay. Irradiated slides were immediately transferred to cold lysis solution and processed through the T4EV-assisted comet assay as described in detail in Chapter 3 and as summarized in Figure 7.2. Experiments were performed in triplicate.

Statistical Analysis

Repeated measures ANOVA was used to investigate the pre-incubation effect of <500EPSCM and HWECMyc on %DNA in comet tails. Results from treated cells were compared to control cells using Dunnett's post- test. Paired t-test was used to investigate differences in response to the two types of extracts. Post-test for linear trend was used to investigate dose response. $P < 0.05$ was regarded as statistically significant. Prism 4.0 (Graphpad Software, San Diego, CA, USA) was used.

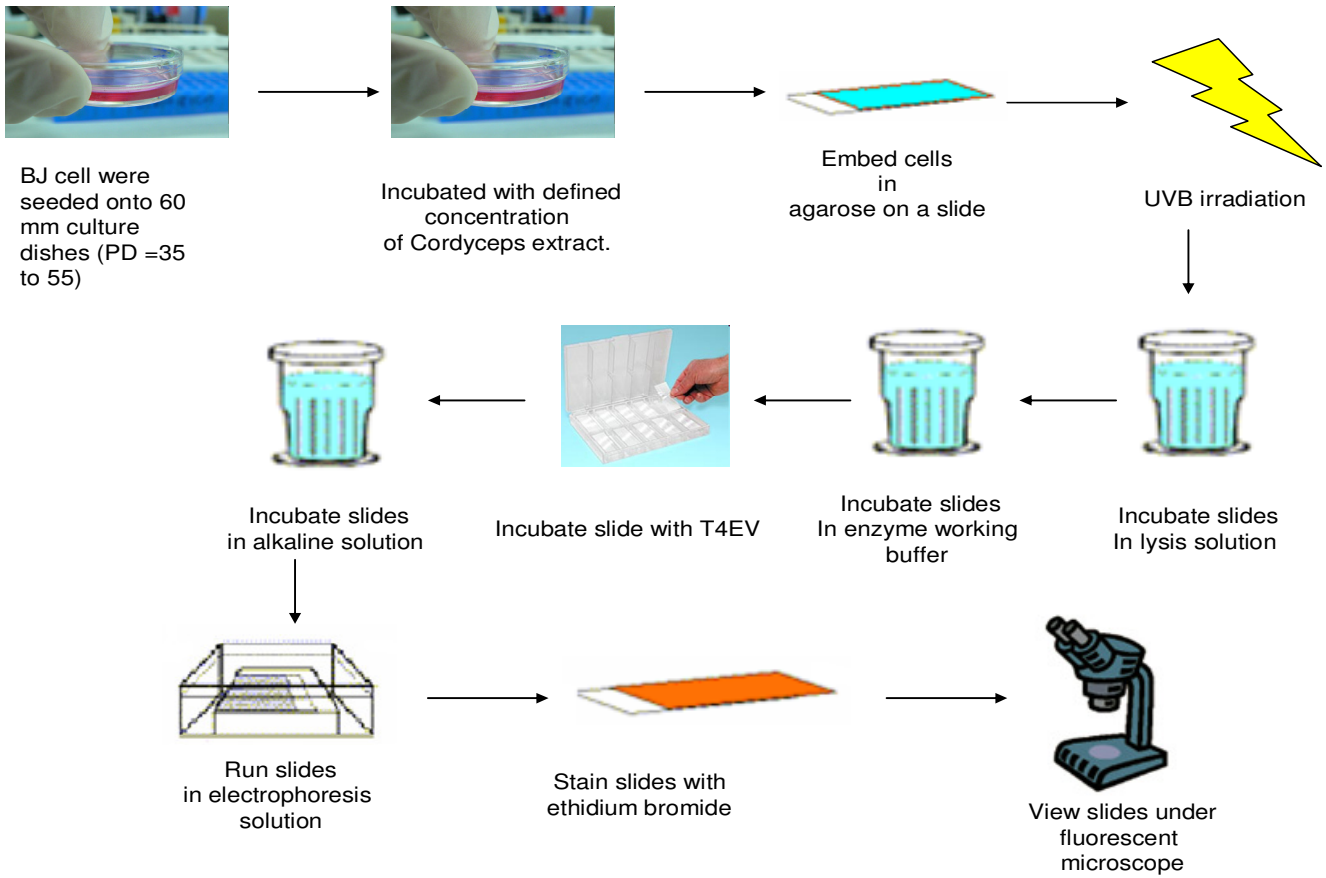

Figure 7.2. Summary of procedures of Cordyceps incubation, UVB irradiation and T4EV-assisted comet assay procedure of BJ cells.

Results

The UV absorption spectra of low gelling point agarose and the Cordyceps extracts are shown in Figure 7.3. Neither of the extracts or the agarose directly absorbed UVB. Neither type of extract was cytotoxic towards BJ cells, at least at up to 200 µg/ml (the highest concentration tested). The viability of cells in triplicate experiments ranged from 92.4 % to 96.1 % after 24 hrs incubation with the Cordyceps extracts. As shown in Figure 7.4, neither type of Cordyceps extract (at 200 µg/ml) induced detectable DNA damage. This figure also shows that without UV irradiation or T4EV enzyme treatment, levels of pre-existing DNA damage in BJ cells were low, and that the background level of CPDs in non-irradiated BJ cells was low also. However, it can be seen that DNA damage, as strand breaks, was 4-5 times higher in T4EV-treated cells after UVB irradiation (Figure 7.4). This highlights the sensitivity of the T4EV-assisted comet assay in detecting UVB-induced DNA damage.

In relation to potential genoprotective effects of the Cordyceps polysaccharides, cells showed significant ($P<0.01$) downward trend in DNA damage with increasing concentrations of each extract for both 30 mins and 24 hrs pre-incubation treatments (Figures 7.5 A,B). A stronger effect was seen for each extract after 24 hrs pre-incubation compared to 30 mins pre-incubation, but 30 mins and 24 hrs results for each extract were not statistically significantly different. The two extracts gave very similar protective effects after both 30 mins and 24 hrs pre-incubation (Figures 7.6 A,B). The protection by each extract at the highest

concentration tested (200 μg/ml) was similar. Compared to complete medium - treated irradiated cells (control), after 30 mins pre-incubation with 200 μg/ml <500EPSCM or HWECMyc, the absolute % DNA in comet tail decreased by 4.2 % and 4.5 % respectively, which was a relative decrease in UVB-induced damage of 20 % and 21.0 %, respectively. After 24 hrs pre-incubation with 200 μg/ml <500EPSCM or HWECMyc, the absolute % DNA in comet tail decreased by 7.2 % and 5.4 % respectively, which was a relative decrease of, respectively, 34 % and 27 %.

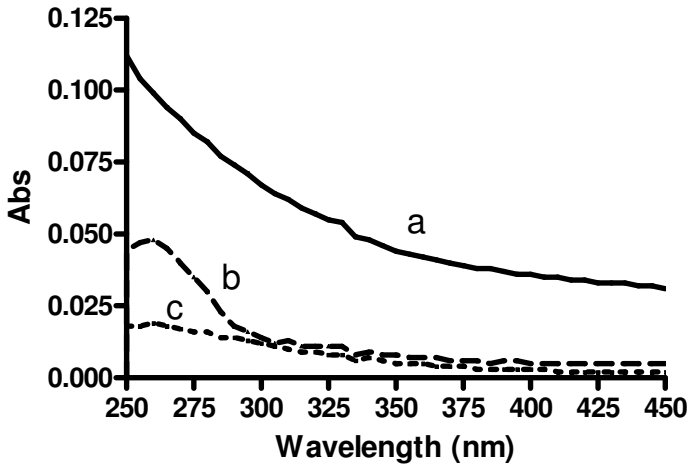

Figure 7.3. UV absorption spectra of (a) 1% low gelling point agarose, (b) 200 μg/ml of HWECMyc, and (c) 200 μg/ml of <500EPSCM. No absorption peak in UVB range (290-320nm) was seen.

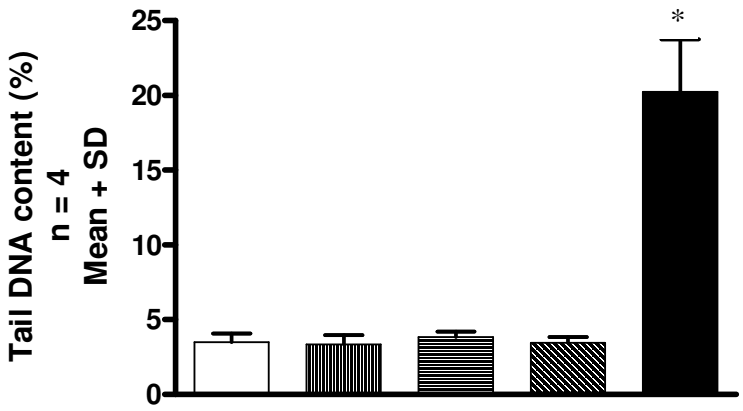

Figure 7.4. The % DNA in comet tail in: cells without irradiation, enzyme or Cordyceps exposure (open bar); cells without irradiation or enzyme but pre-incubated for 24 hrs in 200 μg/ml <500EPSCM Cordyceps extract (vertical lines) or HWECMyc Cordyceps extract (horizontal lines); cells without irradiation or Cordyceps exposure, but with T4EV enzyme treatment (diagonal lines); cells without Cordyceps exposure but irradiated and treated with T4EV enzyme (solid bar): *P<0.01 compared to all other treatments (Dunnett's multiple comparison test after repeated measures ANOVA).

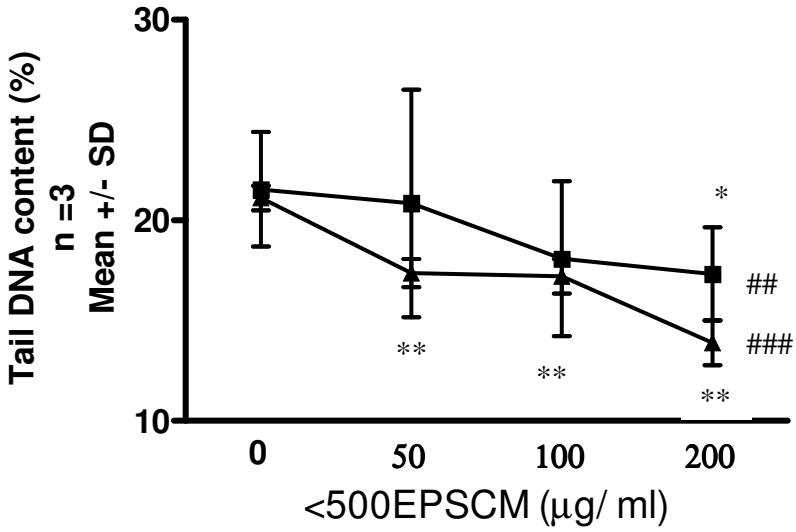

Figure 7.5A. The % DNA in comet tail in UVB irradiated BJ cells after pre-treatment for 30 mins (filled squares) or 24 hrs (filled triangles) with Cordyceps exopolysaccharides extract <500EPSCM. Results are Mean± SD; n=3. Significant protective effect was seen with both 30 mins and 24 hrs incubation. There was a significant protective effect at 200 µg/ml with 30 mins incubation (*P<0.05 by Dunnett's multiple comparison test after repeated measures ANOVA) and at 50-200 µg/ml with 24 hrs incubation (**P<0.01 by Dunnett's multiple comparison test after repeated measures ANOVA). Significant linear dose response was seen with 30 mins Cordyceps pre-treatment (r^2 = 0.22, ## p = 0.007 by post test for linear trend after repeated measures ANOVA) and with 24 hrs Cordyceps pre-treatment (r^2 = 0.84, ### p<0.0001 by post test for linear trend after repeated measures ANOVA).

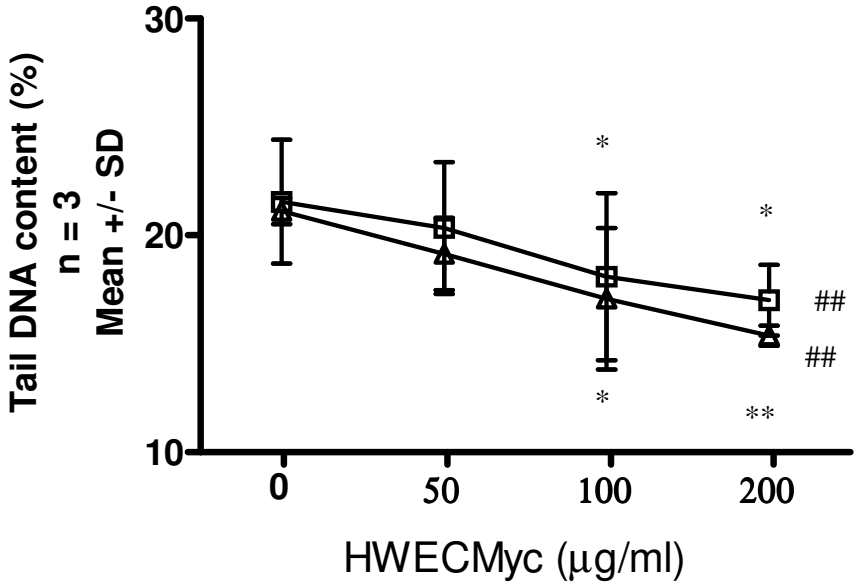

Figure 7.5B. The %DNA in comet tail in UVB irradiated BJ cells after 30 mins (open squares) or 24 hrs (open triangles) pre-treatment with hot water extract of Cordyceps mycelia (HWECMyc). Results are Mean ± SD; n=3. Significant protective effect was seen at 30 mins and 24 hrs incubation. There was a significant protective effect at 100 - 200 µg/ml with 30 mins incubation (*P<0.05 by Dunnett's multiple comparison test after repeated measures ANOVA) and at 100-200 µg/ml with 24 hrs incubation (*P<0.05 and **P<0.01 by Dunnett's multiple comparison test after repeated measures ANOVA) respectively. Significant linear dose response was seen with 30 mins Cordyceps pre-treatment (r^2 = 0.35, ## p = 0.003 by post test for linear trend after repeated measures ANOVA) and with 24 hrs Cordyceps pre-treatment (r^2 = 0.66, ## p = 0.002 by post test for linear trend after repeated measures ANOVA).

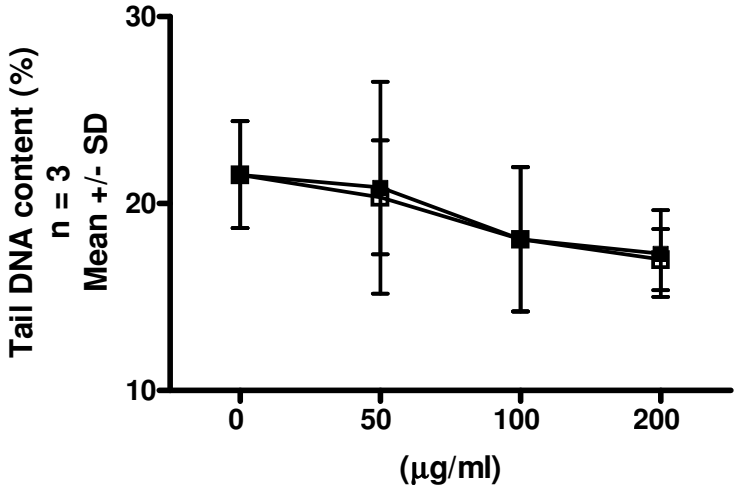

Figure 7.6A. The %DNA in comet tail in UVB irradiated BJ cells after 30 mins pre-incubation with the Cordyceps exopolysaccharides extract (<500EPSCM; filled squares) or hot water extract of Cordyceps mycelia (HWECMyc; open squares). Results are Mean ± SD; n=3. No significant difference was seen between the extracts at any dose tested (paired t test, P >0.05).

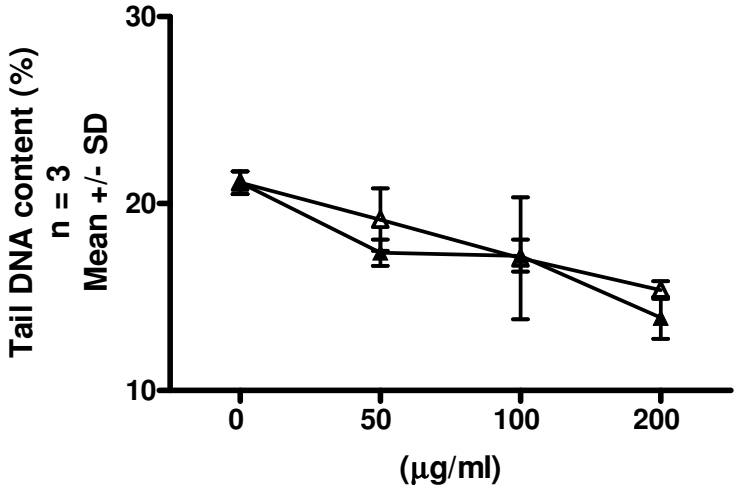

Figure 7.6B. The %DNA in comet tail in UVB irradiated BJ cells after 24 hrs pre-incubation with the Cordyceps exopolysaccharides extract (<500EPSCM; filled triangles) or hot water extract of Cordyceps mycelia (HWECMyc; open triangles). Results are Mean ± SD; n=3. No significant difference was seen between the extracts at any dose tested (paired t test, P >0.05).

Discussion:

UVB exposure induces characteristic DNA lesions, CPDs, leading to mutations and carcinoma of the skin. This is common in older adults and in those who are regularly exposed to UVB by choice or occupation (Situm *et al.*, 2008). Skin cancers are generally non-lethal, but cause disfigurement and can be costly to treat. CPDs also associate with melanoma, which is difficult to treat, often has a rapid progression and spread, has high fatality rate, and rates are increasing (de Braud *et al.*, 2003). Effective prevention strategies for both melanoma and non-melanoma skin cancers are needed. These should not be limited to chemical-based barrier sunscreens, which have their own disadvantages in relation to allergy,

efficacy and safety (Goossens, 2004). Consequently, there is increasing attention being paid to photoprotective effects of natural products. For example, green tea polyphenols have powerful antioxidant properties and absorb UV light (Tobi *et al.*, 2002; Morley *et al.*, 2005). This indicates that tea polyphenols might be useful sunscreens and help prevent oxidation-induced DNA damage. However, tea polyphenols generate hydrogen peroxide *in vitro*, and can irritate skin (Chai *et al.*, 2003). Tea polysaccharides, which do not absorb UV light, were found to maintain moisture levels and decrease damage in UV irradiated cells (Wei *et al.*, 2009). No study to date has investigated polysaccharides in relation to DNA protection against photo-induced DNA damage, although polysaccharides from various natural products, including Cordyceps, are reported to be anti-tumourigenic (Wachtel-Galor *et al.*, 2004a; Ng & Wang, 2005; Leung *et al.*, 2006; Tao *et al.*, 2006; Wu *et al.*, 2007).

Natural Cordyceps is rare, and sources cannot meet the high worldwide demand for this 'herb'. Most of the Cordyceps materials available, including the ones tested in this study, are from cultured mycelia of Cordyceps. Cordyceps contains a wide range of bioactive constituents including mono-, di-, and poly-saccharides, nucleosides, protein, amino acids, trace elements, vitamins E, K, B1, B2 and B12 and sterols (Holliday & Cleaver, 2008). Polysaccharides are one of the major components in Cordyceps, accounting for 3-8 % of dry weight of Cordyceps mycelium, and polysaccharides are also secreted into the culture medium (and termed 'exopolysaccharides'). Cordyceps polysaccharides are reported to have

antioxidant, anti-tumour and immunomodulating effects (Paterson, 2008; Zhou *et al.*, 2009). It was suggested that, besides the pharmacological effect of Cordyceps polysaccharides, their physicochemical properties, such as thickening, good solubility in water and good stability over a wide pH range, give complementary advantages for its use as an additive in sunscreen products (Zhou *et al.*, 2009).

In this study we found no UV absorption by Cordyceps polysaccharides, and so it is unlikely to be a useful sunscreen. However, results of this study have revealed that Cordyceps polysaccharides had a significant protective effect against UVB-induced DNA lesions. After 24 hrs pre-incubation of human skin fibroblasts in 200 µg/ml polysaccharides-rich extracts of Cordyceps mycelia, there was ~30 % less damage in UVB irradiated cells compared to control cells. This has important implications for use of Cordyceps polysaccharides as topical agents or possibly as dietary supplements for protection against UVB-associated skin cancers.

While significant DNA protective effects against UVB were seen in this study, results must be regarded as preliminary. The extracts were polysaccharide-rich, but were crude uncharacterized extracts, and further work, including activity-led fractionation, is needed to identify the bioactive polysaccharides. In addition, the mechanism of action is not clear. The Cordyceps extracts did not absorb UV directly and, as the cells were lysed immediately after UV irradiation, the lower DNA damage with Cordyceps was unlikely to be related to increased DNA repair. Results showed effects were more marked after 24 hrs pre-incubation with Cordyceps,

compared to after 30 mins pre-incubation. This time effect may be related to higher intracellular content of Cordyceps polysaccharides after 24 hrs, could be due to time needed for biotransformation of Cordyceps polysaccharides within the cell, or could be due to a Cordyceps-triggered genoprotective cellular adaptation. The reflective ability of extracts cannot be excluded, and it is possible that the larger molecular weight polysaccharides may be able to adhere to the cell membrane and reflect a part of the UVB radiation, lowering the amount reaching the DNA.

In this study we used an enzyme assisted comet assay to measure UVB-induced DNA damage. The enzyme T4EV is a DNA repair enzyme that recognizes CPDs, and is responsible for the initial step of repair of the CPD lesion. T4EV identifies CPDs in double strand DNA, and cleaves the N-glycosylic bond of the 5'-pyrimidine of the dimer and the 3' phosphodiester bond, resulting in an abasic site, which can be visualized and quantified by using the comet assay (Morikawa *et al.*, 1994; Lloyd, 2005). This T4EV-assisted comet assay offers a useful tool to study UVB induced DNA photoproducts (Sauvaigo *et al.*, 1998; Sastre *et al.*, 2001; Danaee *et al.*, 2004). However, the enzyme is not entirely lesion-specific. T4EV also has AP endonuclease activity (Cafardi & Elmets, 2008). To investigate if Cordyceps can protect against oxidation-induced DNA damage (such as is caused by exposure to reactive oxygen species produced by, for example, UVA radiation and inflammation), other enzymes that recognize oxidation-induced lesions can be used in the comet assay. These include Fpg and Endo III (Collins & Horváthová, 2001). Kielbassa *et al* showed the formation of Fpg sensitive sites is nearly constant

in irradiated Chinese hamster ovary cells over the UVB range (290-315 nm) (Kielbassa *et al.*, 1997). However, UVB-induced damage is predominantly non-oxidative. The rate of CPDs formation with UVB exposure is about 200 times that of Fpg sensitive sites (Kuluncsics *et al.*, 1999), and the formation of Endo III sensitive sites is only about 1 % that of CPDs (Kielbassa *et al.*, 1997). In preliminary studies we tested the Cordyceps extracts for total antioxidant content using the Ferric Reducing/Antioxidant Power (FRAP) assay (Benzie & Strain, 1996) and found it to be low; 54 µmol/g for the < 500EPSCM extract and 28 µmol/g for the HWECMyc extract. Therefore, we suggest it is unlikely that the protective effects of Cordyceps polysaccharides against UVB-induced DNA damage was due to direct antioxidant effects, because A) antioxidant power was low and B) the damage assessed is not oxidation-induced.

The damage induced in this study was limited to that caused directly and rapidly by UVB, as cells were irradiated for a very short time (10 secs) and then immediately lysed. Interestingly, additional post-irradiation damage is also reported to induce CPDs by metabolic changes that involve a nitric oxide-mediated pathway (Wong *et al.*, 2004). This is evidenced by less damage in cells kept at 4 ^{o}C, by protective effects of an inhibitor of inducible nitric oxide synthase, and by the production of nitrates in cells in the hours following UVB irradiation. Further study is needed to reveal if Cordyceps can also modulate this post-irradiation damage, or if the immediately protective action of Cordyceps prevents the post-irradiation events.

In conclusion, results of this study provide the first evidence that polysaccharide-rich extracts from Cordyceps mycelia can protect DNA in human skin cells against UVB radiation. This is important because the UVB interacts directly with DNA to create characteristic lesions, the CPDs, that are mutagenic and associated with both melanoma and non-melanoma skin cancers. The mechanism of protection and key bioactives within the polysaccharide-rich extracts are not yet known. However, on the basis of the work presented here, the significant DNA protection with Cordyceps polysaccharides is unlikely to be due to direct absorption of UVB (although reflection cannot be ruled out), to direct antioxidant action, or to upregulation of DNA repair. Effects may be due to genoprotective adaptations in the cell, or to intracellular biotransformation of Cordyceps polysaccharides. Further study of these interesting initial findings is needed.

Data presented in this chapter have resubmitted with minor revisions after initial review:

Photoprotective potential of cordyceps polysaccharides against ultraviolet B radiation-induced DNA damage to human skin cells. *J Am Acad Dermatol* 2010.

(with Wu JY, Benzie IFF)

Chapter 8

SUMMARY OF MAIN FINDINGS AND CONCLUSIONS, LIMITATIONS AND SUGGESTIONS FOR FURTHER STUDY

The association between increased oxidative stress and many age-related diseases, such as cancer, diabetes, and cardiovascular diseases, is established (Halliwell, 2007; Halliwell & Gutteridge, 2007; Choi *et al.,* 2008). This is becoming a key issue in developed countries since increased lifespan is accompanied with increased healthcare burden (Yach *et al.*, 2004; Chan *et al.*, 2007). The mechanisms of aged-related disease are still unclear, but a key factor is believed to be oxidatively modified biomolecules, such as DNA, protein and lipid (Cooke *et al.*, 2003; Halliwell & Gutteridge, 2007). DNA contains the genetic material responsible for maintaining normal metabolism. Therefore, maintenance of DNA integrity is a key factor for health promotion and accordingly decreasing risk of age related diseases. It may be possible to achieve this by increasing antioxidant defence against damage, and by promoting DNA repair. Conceptually, increased intake of antioxidants can decrease oxidatively induced damage. However, intake of large doses of 'pure' antioxidant may have adverse effects (Omenn *et al.*, 1996; Goralczyk, 2009). A more simple, safe and economic way to increase the body antioxidant defence is by increased intake of food high in antioxidants rather than antioxidant supplements. Therefore, in this study, 'whole food' and extracts made from an entire 'herb' were used to investigate their genoprotective effects.

In this study, the genoprotective effects of Lingzhi, green tea, Cordyceps and bilberry were the main focus of interest. These functional foods were chosen due to their high reputation in health promotion. Lingzhi and Cordyceps have been used in China as 'functional foods' or health promoting 'herbs' to maintain well-being and for disease prevention for thousands of years (Wachtel-Galor *et al.*, 2004b; Wachtel-Galor *et al.*, 2004c; Leung *et al.*, 2006; Paterson, 2008). Green tea is rich in catechins and is commonly consumed as a beverage in Asian countries where it has high reputation for health promotion (Williamson & Manach, 2005; Khan & Mukhtar, 2007). Bilberry is rich in anthocyanins and is associated with hypoglycaemic effects and vision improvement (Canter & Ernst, 2004; Matsui *et al.*, 2006). However, there is a lack of scientific research to support their use for health promotion, and this inspired the current study. Each part of this study had a different experimental design in order to investigate different mechanisms involved in genoprotection. Various versions of the comet assay were used as the core technique to match with different purposes in each part of study.

The focus of the Lingzhi study was to investigate the acute post-ingestion effect on lymphocytic DNA and plasma antioxidant level in healthy subjects. The work was an extension of previous work by our group, which showed background DNA level and DNA damage induced by H_2O_2 did not show significant change after a single dose of Lingzhi extract (3.3g) (Wachtel-Galor *et al.*, 2004b). This work complemented that study because lymphocytic DNA repair capacity was not assessed (Wachtel-Galor *et al.*, 2004b). Here, the background DNA damage level (as single strand breaks), the ability to withstand a standard H_2O_2 –induced

oxidative challenge and repair of damage induced by this challenge in lymphocytes pre- and post Lingzhi ingestion were demonstrated by three different versions of comet assay. The plasma antioxidant level was measured by the FRAP assay (Benzie & Strain, 1996). Results indicated that a single moderately high dose of Lingzhi (3.3g, equivalent to 8.5 capsules) was not associated with any significant change in the biomarkers measured. Results in this study showed that the FRAP value was seen to increase quickly after Lingzhi ingestion, but results varied widely and the increase was small and not statistically significant. No changes were seen in any of the DNA data from the comet assays. Results from our previous study of 28 days' supplementation (Wachtel-Galor *et al.*, 2004c) showed no effect on DNA resistance to challenge, but repair was not assessed. Since the DNA repair system is a combination of various mechanisms and involves different enzymes, a longer time of days or weeks of regular intake may be needed for DNA repair enzymes to be upregulated. The data on time related DNA repair presented here will be useful in planning larger and longer term studies for this purpose.

The focus of the bilberry study was to investigate the effect of bilberry extract on the BER pathway in a group of Type 2 DM patients by using the cell extraction version of the comet assay. This assay mainly assesses hOGG1 activity, which is the first step in the BER pathways (Collins *et al.*, 2001b; Collins *et al.*, 2008). This work was done because our group has previously demonstrated increased DNA damage in Type 2 DM patients (Choi *et al.*, 2005), and previous study shows that this correlates with increased oxidative stress, depleted antioxidant

defence and decreased DNA repair (Blasiak *et al.*, 2004; Choi *et al.*, 2005). Bilberry has been reported to lower glucose (Matsui *et al.*, 2006) and it was of interest to see if it could improve DNA repair. A study of this type has not been performed before, and has important implications if positive results could be demonstrated, as the prevalence of type 2 DM is very high in Hong Kong (Choi *et al.*, 2005). However, results of this part of study showed that 4 weeks of anthocyanin-rich bilberry extract did not increase hOGG1 activity. Furthermore, hOGG1 activity showed no significant relationship neither with fasting plasma ascorbic acid nor glycaemic control. The lack of effects and relationship in this preliminary study may be due to small sample size, low bioavailability of anthocyanins and reflect that subjects were under good glycaemic control. However, regular intake of low bioavailability agent may have some health beneficial effects in long term. The hOGG1 activity data obtained in this study are useful in planning a larger and longer term supplementation study with Type 2 DM subjects with different severity in glycaemic control and oxidative stress. Then we can elucidate whether anthocyanins-rich bilberry has any effect on hOGG1 in relation to the severity of glycaemic control and oxidative stress.

The focus of green tea study was to investigate the genoprotective effect of 4 weeks supplementation of green tea on urine 8-oxodG (from nucleotide pool oxidation, as measured by LCMS) and lymphocytic 8-oxoGua (nuclear DNA oxidation, as measured by Fpg-assisted comet assay). In the planning stage of the study, we believed that the urine 8-oxodG was a biomarker of DNA repair, as many

papers has presented it as this (reviewed by Møller & Loft, 2006). Therefore, the original plan of this study was to investigate the relationship between urine 8-oxodG and lymphocytic 8-oxoGua. The rationale behind this was that the level of oxidatively modified DNA within cells (to be assessed by the Fpg-assisted comet assay) is the dynamic equilibrium point between the rate of DNA damage and the rate of repair (Halliwell, 2000). By looking at pattern of change in urine 8-oxodG and Fpg-assisted comet assay after green tea we would (we thought) be able to discern if effects were mainly through DNA protection (DNA damage down, no effect on 8-oxodG in urine), or if it involved increased repair (urine 8-oxodG up, DNA damage down). However, during the course of the study it became more clear that the origin of urine 8-oxodG is from sanitation of the nucleotide pool, rather than oxidized lesions removed from nuclear DNA (Cooke *et al.*, 2008). Therefore, results from this part of the study demonstrate the effect of green tea supplementation on 'whole body' oxidative stress (urine 8-oxodG) and oxidation-induced damage to nuclear DNA. In this part of the study, results showed green tea had significant protective effects at the nuclear DNA level, but did not appear to affect whole body oxidative stress. This may be due to targeting of action of bioactive components towards DNA. This effect could be direct antioxidant effect if, for example, the tea polyphenols bind to DNA, or could be an indirect cytoprotective effect, for example by adaptations to a small pro-oxidant change in redox tone induced by green tea polyphenols (Halliwell, 2008; Benzie & Wachtel-Galor, 2010). Further study of this concept is needed. It has to be noted also that biological variation in urine 8-oxodG is high (Lee *et al.*, 2010), and it may be that a larger study is needed to demonstrated

supplementation related changes in this biomarker. Nonetheless, this current study is the first controlled human trial to investigate effects of green tea on oxidation-induced DNA damage and 8-oxodG in controlled human study, and the first to demonstrate significant genoprotective effects of green tea. In addition, this study is the first to use two types of green tea. Interestingly, each tea gave similar genoprotective results. This may indicate results from this study can be applied to any kind of green teas in general.

The focus of the Cordyceps study was to investigate the photoprotective effect of cultured mycelia of Cordyceps extract against UVB-induced DNA damage. Normal human fibroblasts were used in this *in vitro* study. Results in this part of the study are the first to show that polysaccharides rich Cordyceps extracts can significantly protect human fibroblasts against UVB-induced damage. The mechanism behind the effect is not yet known. However,it does not appear to be due to direct UVB absorption, since, there was no absorption peak of either of the Cordyceps extracts tested in the UVB region. Also, it is unlikely that less DNA damage in irradiated Cordyceps-treated cells was related to increased DNA repair because the fibroblasts were lysed immediately after UV irradiation, allowing no time for repair. Furthermore, results showed greater levels of protection after 24 hrs pre-incubation with Cordyceps, when compared to the same concentration at the 30 mins time point. This may be due to higher intracellular concentration of Cordyceps accumulating in the fibroblasts, or could be due to biotransformation of Cordyceps, or due to genoprotective cellular adaptation triggered by Cordyceps. Further study

of mechanisms is needed. However, the results have potential importance because UVB can directly react with DNA and resulting mutagenic DNA photoproducts, the CPDs and 6-4 PPs.

Strengths And Limitations Of The Study, And Suggestions For Future Research

This study had several strengths. The Lingzhi (Chapter 4), green tea (Chapter 5) and bilberry (Chapter 6) studies were controlled studies with cross-over design. This means each subject acted as their control, and can increase the power in each study, even though the sample numbers were small (due to resource limitations). Also, the doses used were not high, and were chosen to represent what would be taken in normal dietary practice. In the Cordyceps study (Chapter 7), a normal human fibroblast cell line (BJ) was used. This was chosen to try to emulate normal skin cells, the tissue of interest for UVB damage, which causes skin cancer (Marrot & Meunier, 2008). The BJ cell line has a longer lifespan compared with other normal human fibroblast cell lines, but is reported to have a normal karyotype for up to 61 population doublings (Morales *et al.*, 1999). In the Cordyceps study, all cells tested had a population doubling number of 35-55, and all experiments were carefully controlled. Therefore, we can assume cells used are basically under same conditions, and the result obtained reflects the protective effects of Cordyceps rather than due to the change in the cells' condition.

In this study, various versions of comet assay were adopted for assessing different aspects of possible genoprotective effects of four different functional foods. Ideally, this study should focus on a single functional food and investigate the geneoprotective effects in various aspects by using different versions of comet assay. However, time was needed to set up the different versions of comet assay and we were limited in the volume of blood we could sample from our human volunteers. In particular, the cell extract version of the comet assay for DNA repair requires quite a large volume of blood. This is why in the bilberry study we could not also measure the pre-existing DNA damage using the Fpg-assisted comet assay as well, which would have been useful, and why we could not do the cell extract version in the other two supplementation studies. Also, it was not feasible for one researcher to finish all the set up and optimization and perform all versions of the comet assay in a single study due to time limitations, as the comet assay is quite a labour intensive test. Therefore, we decided to perform a series of smaller supplementation studies based on our previous work, and following the progress of comet assay set up in our laboratory, which was, standard alkaline version, then the H_2O_2 challenge version, the timed repair version after challenge, the Fpg-assisted version, the T4EV-assisted version, and finally the cell extract version.

Another limitation is the sample size in the Lingzhi, green tea and bilberry supplementation studies. Sample size was based on the budget, manpower, the duration of each trial and feasibility of recrutiment. Manpower is the main concern in our team, especially in the Lingzhi supplementation study. In that part of the

study, fresh lymphocytes were used to run the timed repair version of comet assay. This investigator was responsible for harvesting lymphocytes and running the challenge and timed repair versions of comet assay, and only small numbers of sample could be handled.

A cell culture method was adopted in the Cordyceps study. Crude extracts from mycelium culture were kindly supplied by a collaborator (Dr JY Wu of the Department of Applied Biology and Chemical Technology of PolyU). These extracts are known to be rich in polysaccharides, but have not been further characterized. The results obtained in this *in vitro* study may not translate into physiological effects in topical application or supplementation trials, but offer interesting evidence of protection that will justify future animal and human studies. However, it is noted that only one batch of Cordyceps extracts was tested, and results obtained in this study need to be reconfirmed with other batches of extracts with same experimental setting, and testing of commercial Cordyceps products would also be useful in follow-up studies.

Future research is needed also to identify bioactive constituents. It is assumed that the benefits of green teas are related to its polyphenols. However, in this study we did not measure plasma or urine polyphenols, and in future study that would be useful. There are many biomarkers and biomedical tests that can be applied to investigate health related effects of herbs and to activity guided fractionation of herbs. Chemical fractionation and analysis or characterization of

herbal components followed by determination of bioactive components through cell culture and animal studies are needed. In addition, we need to determine if bioactive components are absorbed (bioavailable), inactivated or created (by biotransformation) after ingestion. The technique of LCMS is increasingly being used for chemical analysis, or 'fingerprinting' of herbal extracts and to identify the bioactive components in plasma or urine. This type of study may help to explain the contradictive results in Lingzhi, bilberry and green tea in the literature. Different versions of the comet assay are the core biomonitoring tool for investigating genoprotection by functional foods. However, there is no single test that can paint the full picture. Various biomarkers such as antioxidant capacity, oxidative stress biomarkers and gene expression, can be included in future research. For example, results obtained in cell extract version of the comet assay mainly demonstrate hOGG1 activity. If antioxidant and oxidative stress biomarkers are measured also the impact of various degrees of oxidative stress on hOGG1 activity can be investigated.

In the Lingzhi study, even though no significant results were obtained, the data obtained can be used as a base for planning further supplementation studies. A larger and longer study with a higher risk group may be needed to observe the beneficial effects of Lingzhi since time may be needed for DNA repair enzymes to upregulate, and a small change in effect may not be easily observed in healthy adults. This herb has a very powerful reputation for health benefits in Chinese tradition, and this merits further study. In the green tea part of the study, a clearer picture on

genoprotective effects can be seen if both the urine 8-oxodG and 8-oxoGua are measured in further study as this would help reveal whether the genoprotective effect is due to direct antioxidant properties or due to increased DNA repair. Furthermore, plasma or cellular level of catechins can be measured, and this allows us to conclude if the protective effect in lymphocytic DNA is due to green tea polyphenols or other components. In the bilberry study, it would be useful to include the lymphocytic Fpg-assisted comet assay. Here, we only investigated the upregulatory effect of bilberry on hOGG1 activity, the enzyme involved in the first step of BER pathway. Even though bilberry did not show a positive effect on hOGG1 activity, we cannot neglect the direct oxidative effect on cellular DNA level. If possible, it would be useful to investigate the relationship of hOGG1 and oxidative stress (using markers such as F2 isoprostanes, allantoin and urine 8-oxodG) and glycaemic control in Type 2 DM subjects. The bioavailability of anthocyanins should be included in further research. In the Cordyceps study, we can further sub-divide the Cordyceps extract into more fractions, and identify which fractions contain the active components responsible for the genoprotective effect. The protective action of Cordyceps on post- irradiation damage also would be of interest.

Concluding Remarks

While the study has several imitations, new data have been presented and, taken together, the different parts of the study demonstrate the application of the various versions of the comet assay, as well as urine 8-oxodG in the assessment of diet and functional foods. On the basis of the results of the different parts of the

study, we have: confirmed that some antioxidants are absorbed from Lingzhi, but these do not appear to affect DNA damage or repair, at least in the few hours after ingestion; shown that bilberry extract in a typical daily dose does not appear to affect hOGG1 activity; demonstrated that regular intake of green tea significantly lowers oxidation-induced damage to nuclear DNA, though it does not appear to affect a biomarker of 'whole body' oxidative stress; demonstrated that Cordyceps polysaccharides protect skin fibroblasts against UVB induced damage.

Various mechanisms are involved in genoprotection, and mechanisms of the effects seen for green tea and Cordyceps are not known. The bioactive components may show a genoprotective effect directly by antioxidant property or by indirectly upregulating the DNA repair capacity or other cytoprotective adaptations. We saw no effects of Lingzhi and bilberry, but we cannot rule out potential benefits of larger doses and/or longer duration of supplementation. In addition, we focused on genoprotection. We believe that less DNA damage will ultimately benefit health, but there are other routes to health promotion, such as through lowering blood pressure, lipids, inflammation, and by improving immune status. Various test methods are needed to investigate the effects of herbs and functional foods. Lack of demonstrable genoprotective effects of a food or herb does not necessarily mean it has no health benefits. Large scale collaborative studies testing a very wide range of biomarkers are needed to fully investigate the health effects and their mechanisms in relation to herbs, teas and other functional foods. It is recommended that, if enough sample is available, several versions of the comet assay (particularly the Fpg-

assisted , the cell extract version, and the T4EV-assisted version for photoprotection if this is of interest) are used, and that urine 8-oxodG be used as a complementary test to assess global oxidative stress. Based on the results presented in this thesis, such follow-up studies on Cordyceps and green tea are warranted.

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Appendix 1

Information Sheet for Volunteers for Research Project Entitled:
Acute effect of ingestion of Lingzhi (*Ganoderma lucidum*) on antioxidant and inflammation markers in human plasma and urine

Principal Investigator: Prof. Iris FF Benzie, Dept of Health Technology and Informatics, The Hong Kong Polytechnic University
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Co Investigators: Dr. Sissi Wachtel-Galor, Dept of Health Technology and Informatics, The Hong Kong Polytechnic University
Tel No. 34008599, e-mail htsissi@

Why is the study being performed?

Lingzhi (*Ganoderma lucidum*), an oriental fungus, is widely used as a remedy for promotion of health and longevity in China, Japan and other Asian countries. It is a popular health food supplement commonly consumed on a regular basis by many Hong Kong Chinese.

Lingzhi is widely used for health promotion, and is believed to have anticancer, immunomodulatory, anti-inflammatory and antioxidant properties.
Our previous research work in human trials has shown that there is a significant increase in the plasma antioxidant and urine total antioxidant power (as the FRAP value) 90 minutes post ingestion of Lingzhi in the form of a commercially available powered product.

This study will use a similar protocol as our previous research work, and the same source and type of commercial powered product will be used. In this study, additional biomarkers for antioxidant and inflammations status will be investigated. In addition to plasma and urine total antioxidant power, changes in urine phenolics and the effect of plasma on phospholipase A2 (enzyme involved in the inflammatory process) inhibition will be examined.

In our previous study, the subjects reported of no side effects during the acute post ingestion study except for the bitter taste of the Lingzhi powder.
Ethical approval for this study has been granted by the Human Subject Ethics Committee of the Hong Kong Polytechnic University.

What is the purpose of the study?
To examine acute effects of ingestion of Lingzhi on plasma and urine antioxidant status, urine total phenolics and plasma phospholipase A_2 (PLA_2) inhibition.

Are there any risks by taking the Lingzhi or participating in this study?
Lingzhi is widely consumed throughout the world as a health food. Local consumption in Hong Kong is high, and its health promoting effects is accepted by many members of the local population. There are no known risks by taking Lingzhi at the planned dose.
In a similar protocol performed by our group, the subjects reported no side effects except for the bitter taste of the Lingzhi powder.
However, it is possible that volunteers may feel nauseous after taking the Lingzhi powder because of its bitter taste.

During the study, three vernous blood samples will be collected from the arm. One sample will be collected before and two will be collected post ingestion of the Lingzhi. Each sample will be 10ml. These will be taken by an experienced phlebotomist, using sterile, disposable syringes and needles.

Therefore there is a minor injury and *volunteers may develop a small bruise on arm at the blood taking site.* The total amount of blood taken on each study day will not exceed 30ml. This volume of blood loss is not associated with any health hazard (in comparison, donating a unit of blood for blood transfusion purposes means you loss 450ml blood). *However, some people do feel faint when their blood is taken.* If this happens to a volunteer in this study, the volunteer should lie down and rest until the feeling passes. The research team will supervise the volunteers during the 3 hours of blood sampling.

What do volunteers for the study have to do?

Volunteers for the study will be asked to:

1 Sign an informed consent form that states he/she understands the information presented on this sheet.

2 Attend our laboratory on pre-arranged dates and times (early morning) and in the fasting state (no breakfast).

3 Give us some information, such as age, health and diet.

4 Tell us about allergies to foods or drinks.

5 Follow the test protocol as described in detail below.

Study Protocol

In this study we will try to examine and identify the absorbable compounds after one time Lingzhi intake. As it is a controlled study, there are 2 parts for the study. In one part of the study volunteers will be asked to take Lingzhi powder together with water and in the other to drink water but without taking Lingzhi.

It should be noted that each subject will attend on two separate days, on one occasion taking the Lingzhi powder plus water, and on the other only water. Except for this, the procedure will be identical on each occasion.

Day 1 (morning)

On the first day of this part of the study, volunteers should arrive at our laboratory at around 9.00 am and in the fasting state i.e. nothing to eat or drink (except water) after 10 pm the night before. A 10 ml venous blood sample will be collected and transferred to a tube. Each volunteer will be asked to provide a urine sample. Volunteers will then be given one of the two treatments, i.e.:

I. 3.3g Lingzhi powder (based on our previous study and within the recommended dose range) with 400 ml water.
II. 400 ml water.
III.

Repeat blood samples (10ml each time) will be collected after 90 and 180 minutes.

Urine will be collected every 90 minutes.

Blood samples will be collected by an experienced phlebotomist and using standard, hygienic procedures, including sterile, disposable needles and syringes. The blood will be taken 3 times (in total).

Plasma or serum from the blood samples and the urine samples will be used to measure antioxidant and inflammation status and to look for evidence of absorption

and excretion of Lingzhi components, especially those with biological effects (such as antioxidant activity or anti-inflammatory effects).

We will also collect the white blood cells from your blood samples and use these to look for Lingzhi-related effects on protection of DNA from damage.

Volunteers will be asked to complete a short questionnaire about age, health status and usual diet and lifestyle.

Day 2 (morning)

Seven days after the end of the first part (day 1), volunteers will return for the same procedure as described above. The difference will be the type of treatment. For example, if in the first part Lingzhi powder with water was taken, in the next part only water will be given, and vice versa.

A short summary

Day 1

As a volunteer:

1. You will arrive fasting to the clinic or lab.
2. Fasting blood sample and urine sample will be collected
3. You will be given one of the two treatments: 3.3g Lingzhi powder with 400 ml water or only 400 ml water.
4. Blood and urine will be collected at regular intervals for the next 3 hours.
5. You will complete a brief questionnaire about your age, health and normal dietary habits
6. Total time per treatment will be around 3.5 hours (9am-12.30pm).

7. Seven days after the end of the first supplement intake, you will repeat the same procedure as described above, but with different treatment. If in ‘Day 1’, you were given Lingzhi in the next time (‘Day 2’) you will be given water and vice versa.

Please, do not start to take any other vitamins or supplements during the study or change your diet or lifestyle; **this is very important**.

We will contact you between the study days by phone or e-mail (as you prefer) to keep in touch.

Please keep as closely as possible to your normal activities during the study. If you are ill, or if you begin a new activity (e.g. exercise class, weight reduction diet) please let us know, as this might influence our results.

In all parts of the study:

If any of your blood or urine is left over we will keep this frozen and use it at a later date for follow-up studies based into nutrition and antioxidants.

If, during the tests, we become aware of any unexpected results on your samples that might give us cause for concern we will contact you.

In all parts of the study, all your samples will be coded, all your information will be kept securely locked away, and personal information will not be revealed to anyone outside the immediate research team unless we have your written permission. You will not be identified or identifiable from any data published or presented by us.

During any part of the study you may withdraw from the study at no penalty.

For more information on the study you can contact:

1. Prof. Iris Benzie, Dept of Health Technology and Informatics, The Hong Kong Polytechnic University, Tel: 34008572; e-mail: htbenzie@
2. Dr. Sissi Wachtel-Galor, Dept of Health Technology and Informatics, The Hong Kong Polytechnic University; Tel: 34008599 or 9238 ; e-mail: htsissi@

We will be happy to answer any questions you may have.

The Ethics Sub-Committee of the Hong Kong Polytechnic University approved this study. However, if you have any complaints about the conduct of this research study, please do not hesitate to contact the Secretary of the Human Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University in person or in writing (Human Resources Office of the University).

CONSENT TO PARTICIPATE IN RESEARCH

Title of Project: Acute effect of ingestion of Lingzhi (*Ganoderma lucidum*) on antioxidant and inflammation markers in human plasma and urine

Principal Investigator: Prof. Iris FF Benzie, Dept of Health Technology and Informatics, The Hong Kong Polytechnic University
Tel No. 34008572, e-mail htbenzie@

Co Investigators: Dr. Sissi Wachtel-Galor, Dept of Health Technology and Informatics, The Hong Kong Polytechnic University
Tel No. 34008599, e-mail htsissi@

I agree to participate in the Lingzhi study (2 mornings):

YES [ ] **NO [ ]**

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.

Date:_____

Name of participant: _____

Signature of participant:_____

Appendix 2

INFORMATION SHEET

To study and compare the effects of two kinds of green tea (Loongjin and Screw-shaped Green Tea) for health promotion, with particular focus on DNA damage, protection and biomarkers of Age-Related Diseases.

You are invited to participate in a study conducted by Professor Iris Benzie, who is a member of academic staff of the Department of Health Technology & Informatics in The Hong Kong Polytechnic University.

1. **Purpose:**
   Due to the aging of our population, healthy aging is an important target for our society. Researchers try to study the cause of age-related diseases so that healthy aging and functional longevity can be promoted.
   The body structures are continuously exposed to potentially damaging reactive oxygen species ('free radicals'). These free radicals are believed to cause oxidative changes leading to the cellular damage and cell death within the body. These changes are counteracted by the antioxidant defences. Dietary supplementation of antioxidants is the trend in the research area and is a possible way to lower risk age-related disease. Green tea is a very rich source of antioxidants, but there are many kinds of green tea on the market. The objective of this project is to study and compare the effects of two different kinds of green tea on biomarkers for DNA oxidative damage, oxidant:antioxidant balance of the body, and risk for cardiovascular diseases.

2. **Method:**
   A randomized, placebo-controlled, single-blinded, human intervention trial of multiple crossover design.

3. **Approach:**
   Various biochemical markers ('biomarkers') of oxidative stress, antioxidant defenses, cardiovascular disease risk, the effects on DNA damage and protection will be measured in fasting blood and urine samples collected pre- and post- 4 weeks supplementation with green tea or water (placebo). Each subject will be allocated to one of three treatments (Loongjin green tea, 'Screw-shaped' green tea or water (as control) for four weeks. A washout period* will follow each intervention study. After the washout period, subjects will be allocated to another treatment for the next 4 weeks. Fasting blood and urine sample will again be collected before and after the second 4 weeks' daily supplementation for measurements of the biomarkers. A second washout period* and third 4 weeks intervention of third supplementation will be followed. Again fasting blood and urine samples will be collected before and after the third intervention study.
   In total, six blood samples and six urine samples will be collected from each subject over the 24 weeks of the study.

   Remarks: * washout period should be at least 6 weeks and not more than 10 weeks.

4. **Outcome, relevance, significance and value:**

The outcome of this work is in relation to improving the protection on oxidative DNA damage, oxidative balance and lowering risk of age-related disease of cardiovascular disease, thereby promoting healthy aging and lowering healthcare costs. Results of this study will be disseminated so as to ensure their uptake in the community we are aiming to help, in addition to dissemination via the usual scientific research channels.

5. <u>What you are asked to do if you volunteer to take part in this study?</u>

If you meet our inclusion and exclusion criteria (given below) you will be asked to:

A. Be part of this study and provide about 10 ml of blood and some urine on SIX mornings over the following 5 to 6 months.
PLEASE UNDERSTAND THIS IS A LONG TERM COMMITMENT (nearly half year).

B. On pre-arranged 'test day' (six in total) mornings we will visit your place or you will come to our workplace or laboratory (as agreed in advance for each test day), before you have your breakfast, and you will then

a. Have about 10ml of blood taken from a vein in your arm
b. Provide a urine sample
c. Be given a 'intervention' supply (either Loongin green tea, Screw-shaped Green Tea or none as placebo) to take home with you
d. Take 'green tea' (or water) in each morning and evening every day for the next 4 weeks as instructed
e. Return fasting (no breakfast and no supplement on that day) on a pre-arranged date and time (after the first 4 weeks intervention) for the procedures in a) to b) to be repeated
f. Not take any green tea in the following washout period*
g. Return on a pre-arranged date and time (after the 6 weeks washout period) for the procedures in a) to d) to be repeated with second 'intervention'
h. Return on a pre-arranged date and time (after the second 4 weeks intervention) for the procedures in a) to b) to be repeated
i. Not take any green tea in the following second washout period*
j. Return on a pre-arranged date and time (after the 6 weeks washout period) for the procedures in a) to d) to be repeated with third 'intervention'
k. Return on a pre-arranged date and time (after the third 4 weeks intervention) for the procedures in a) to b) to be repeated

Remarks: * washout period should be at least 6 weeks and not more than 10 weeks.

IT IS EXTREMELY IMPORTANT THAT YOU DO NOT KNOW WHAT KIND OF GREEN TEA YOU ARE TAKING IN EACH INTERVENTION. YOU WILL ONLY BE ABLE TO KNOW THAT ONE OF THE INTERVENTIONS IS THE PLACEBO (WATER) CONTROL. IT IS ALSO VERY IMPORTANT THAT YOU WILL FOLLOW YOUR USUAL DAILY DIETARY INTAKE DURING THE WHOLE INTERVENTION STUDY. OUR GREEN TEA SUPPLEMENTATION ONLY ADDS ON YOUR NORMAL USUAL DAILY DIETARY INTAKE BUT NOT TO REPLACE THEM.

On the first 'test day' morning we will ask you to give your written consent and to provide some personal information (e.g. age, smoking history, history of illness, food supplements you take, some other dietary information) and have your blood pressure, weight, height, hips and waist measured. We will take a fasting blood and urine sample, and we will give you one type of 'green tea' (or nothing for placebo control) to take each day for the next 4 weeks.

During the whole study period, we will keep in regular contact with you (at least once a week) by

phone or e-mail. If you want to contact us at any time you are welcomed to do so, and we will provide contact fax, phone and e-mail details on the first test day morning.

6. What is the risk of taking green tea?

There is no significance risk in taking green tea. The dosage we use is the normal concentration in general daily intake.

7. What is the risk of blood sampling?

Experienced nursing or technical staff will take your venous blood. In some cases, a little bruise may appear around the phlebotomy area. There is also a possible infection risk after drawing blood. However, sterile and disposable needles will be used to reduce the chance of infection.

8. What will we do with your blood and urine samples?

Firstly we will code them so that your name is not on them (only the research team will be able to break the code). This is to keep your information confidential. Then we will perform some laboratory tests of biomarkers in your samples. We will assess your oxidant: antioxidant balance, risk for cardiovascular diseases and degree of DNA damage of your lymphocytes change after intake of 'green tea' or 'water using a biomarker approach. This will give us scientific information on how the human body responds to these supplements in terms of the biomarkers of oxidative status and the age-related diseases.

9A To be a volunteer on this study we need you to be able to say 'yes' to these points:

Aged 35-55 years; apparently healthy; non-smoker; with no serious hypertension (<160mm Hg systolic and <105mm Hg diastolic pressure) with or without standard hypertensive treatment; no more than mild to moderate hyperlipidaemia (we will measure your total cholesterol and triglycerides on the fasting sample taken on the first 'testing day'. If we find your cholesterol or triglycerides levels are too high, we will inform you if you would like us to, as it would be advisable for you to have these checked and followed up by your own doctor).

9B We also need you to be able to say 'no' to these points:

With previous history of stroke, heart attack (myocardial infarction) or angina; diabetes mellitus; cancer; immune disorder; illness that has required hospitalization during previous 12 months (confined to Medical Ward); severe obesity; blood pressure >160/105mm Hg; under medical prescribed diet; under slimming regime; under regular medication including TCM and pregnant or lactation women.

Even after you volunteer, you have every right to withdraw from the study before or during the trial without penalty of any kind. All information related to you will remain confidential, and will be identifiable by codes known only to the researcher.
If you have any complaints about the conduct of this research study, please do not hesitate to contact Mr. Eric Chan, Secretary of the Human Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University in person, telephone number 27665315 or in writing (c/o Human Resources Office in Room M1303 of the University).

If you would like more information about this study, please contact **Professor Iris Benzie at Tel. no. 34008572** or htbenzie@

Thank you for your interest in participating in this study.

WRITTEN CONSENT TO PARTICIPATE IN RESEARCH PROJECT ENTITLED:

To study and compare the effects of two kinds of green tea (Loongjin and Screw shaped green tea) for health promotion, with particular focus on DNA damage and protection, and biomarkers of age-related disease

I _____ hereby consent to participate in the captioned research conducted by Han Kam Chu, Rita Lo, Angela Wong under the supervision of Professor Iris Benzie.

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 procedures as set out in the attached information sheet have been fully explained. I understand the benefits 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.

I would / would not *like to be informed if abnormal results are found during the screening of my blood or urine sample.

I volunteer to take part in this study.

Name of participant _____

Signature of participant _____

Name of _____

Signature of researcher _____

Date _____

***Please indicate your choice**

Appendix 3

INFORMATION SHEET FOR VOLUNTEERS FOR RESEARCH PROJECT ENTITLED:

Evaluation of Bilberry (*Vaccinium myrtillus*) Supplementation on Biomarkers of Glycaemic Control, Antioxidant Status, Inflammation and Coronary Heart Disease Risk in Type 2 Diabetic Subjects

The key points in relation to this study are below. Please read them carefully and feel free to ask for clarification on any points or procedures you do not fully understand.

Key points:

1. Type 2 diabetes is very common in Hong Kong and often leads to inflammation and damage to blood vessels, and to depleted antioxidant defences in diabetic patients
2. These changes lead to serious complications such as kidney damage, heart disease or stroke
3. Current management/treatment strategies cannot prevent these complications, but good control of blood glucose is crucial to improving health and lowering risk of complications in diabetes
4. Increased antioxidant defence may help lower oxidative stress in type 2 diabetes and could help lower complication risk
5. There is some evidence that anthocyanins, polyphenolic compounds found in blue/red/purple berries, have anti-inflammatory and glucose lowering effects, as well as being powerful antioxidants
6. Bilberry (*Vaccinium myrtillus)* is one of the richest natural sources of anthyocyanins
7. If beneficial effects (antioxidant, anti-inflammatory, glucose lowering)f bilberry are confirmed, increased intake of bilberry could bring significant health benefit to type 2 diabetes patients

Study Aim: to investigate the effect of bilberry supplementation on glucose levels, antioxidant status, inflammatory status in type 2 diabetic subjects.

Criteria to become a volunteer in this study:

To be a volunteer on this study, we need you to be able to say 'yes' to these points:

- Aged between 30-70 years
- Diagnosed with type 2 diabetes for at least 1 year
- Non-smoker
- In good general health apart from the diabetes or other related chronic stable conditions such as hypertension and dyslipidaemia
- No history of heart disease or stroke
- No hospitalization within the past year
- No taking any vitamin or antioxidant supplements or Chinese medicine during the course of the study

This is what you will be asked to do if you become a volunteer in this study:

- Arrive at the clinic at a preset time (around 9am) and date in *the fasting state* (nothing to eat or drink except water since around 10pm the previous evening) and provide us with a few personal details (age, body weight and height).
- About 20 ml of blood samples will be drawn from a vein in your arm by an experienced research staff.
- You will also be asked to provide a urine sample.
- After that, you will be given a bottle of capsules and you will take 2 capsules with a drink of water every morning and every evening for 4 weeks. The capsules contain either bilberry extract powder or a placebo ('dummy') powder.
- At the end of 4 weeks, you will come back to the clinic and repeat fasting blood samples and urine samples will be collected again. Note – you will not take any capsules that morning before blood taking.
- For the next 6 weeks your will not take any capsules.
- After this 6 weeks period with no capsules you will come back and repeat the blood and urine sampling (again in the fasting state) and will be given the other set of capsules. Again you will take 2 capsules with a drink of water every morning and every evening for 4 weeks. Everything is the same in this second 4 week period except that if you had been given bilberry extract supplement at the start of the study, you will be

given placebo capsules this time, and vice versa. It is important that you do not know which you are taking. It is important also that you keep to your usual diet and lifestyle so that any differences we detect in health markers in your blood or urine are due to the capsules and not to changes in other factors.

- Your part in the study will last 14 weeks.

This is what we will we do with your samples

Your sample will be assigned a study code ID and your private information will be kept private and under confidential cover, and accessible only to the research team.

Your blood sample and urine will be tested to assess glucose control, inflammation, immune status, antioxidant balance and heart disease risk.

What are the risks and benefits to you if you volunteer for this study?

Risks: We do not expect any harmful side-effects from you taking 4 capsules of the bilberry extract per day as there is no scientific evidence of harmful effects of bilberry supplementation on health. Even at doses many times higher that in this study no harmful side-effects of toxicity has been reported. As with ingestion of other fruit extracts, some people may experience gastrointestinal discomfort, and there is a chance that the fruit extract could affect absorption of your usual medications. *Therefore, we ask you to avoid taking the bilberry capsules at the same time as other medications you take by mouth.*

The blood collection should not result in any undue discomfort, although you may suffer a small bruise over the blood taking site. This will only last a day or two. Risk of infection is very small because we use sterile disposable needle and the blood taking is performed by an experienced research staff.

If you experience any side-effects you think are due to the taking the capsules please:

1) stop taking them
2) contact us as soon as possible to let us know (use the number below)

Benefits: You may not have any benefit by becoming a volunteer in this study. However, the results of the study will help us to understand the short term effects of bilberry supplementation on health status and in type 2 diabetic patients and the potential effects of bilberry on lowering the risks of diabetic complications.

What are your rights?

If you become a volunteer, you have every right to withdraw from the study at any time without penalty of any kind.

You have every right to ask questions of us, and we will answer these truthfully and in terms you can understand.

During the whole project, you will be identified only by a code, which will only be known by our research team. You will not be able to be identified by any information published or presented by the study team.

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

If you would like more information about this study, please contact

Prof Brian Tomlinson
Tel: 2632
Ms Chau, Ms Yeung, Ms Chu
Tel: 2632

Thank you for your interest in participating in this study.

WRITTEN CONSENT TO PARTICIPATE IN RESEARCH PROJECT ENTITLED

Evaluation of Bilberry (*Vaccinium myrtillus*)
Supplementation on Biomarkers of Glycaemic Control,
Antioxidant Status, Inflammation and Coronary Heart
Disease Risk in Type 2 Diabetic Subjects

I _____ (HKID:_____)hereby consent to participate in the captioned research conducted by Prof Brian Tomlinson.

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 procedures as set out in the attached information sheet have been fully explained.

I understand the benefits 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.

Signature of patient/date 自願參加者簽署 / 日期

……

Investigator:研究者：

I hereby state that I have fully explained the nature, purpose and possible risks of participation in this research to the above signed

本人聲明已向病人詳細解釋此研究的性質、目的及可能引起的危險。
Name of investigator 研究者姓名
Signature of investigator/date 研究者簽署

Appendix 4

桂林莱茵生物科技股份有限公司
总部：桂林市漓江路 22 号桂林国际会展中心 A 区 3 层 541004
厂部：桂林兴安县湘江路 18 号，541300
电话：+86-773-5878095　　传真：+86-773-5878080
电子信箱：info@layn.com.cn 网址：www.layn.com.cn

Certificate of Analysis

Product Name:	**European Bilberry P.E.**	**Manufacture Date:**	**2007-JAN-08**
Latin Name:	**Vaccinium Myrtillus**	**Testing Date:**	**2007-JAN-08**
Batch Number:	**BIL01-070101**	**Expire Date:**	**2009-JAN-07**
Quantity:	**200kgs**	**Shelf Life:**	**2 Years**

ITEM	SPECIFICATION	TEST RESULT
PHYSICAL TESTS:		
DESCRIPTION:		
APPEARANCE	DEEP PURPLE POWDER	COMPLIES
ODOR	CHARACTERISTIC	COMPLIES
TASTE	CHARACTERISTIC	COMPLIES
COUNTRY OF ORIGIN	EUROPE	COMPLIES
PARTICLE SIZE	THROUGH 80 MESH	COMPLIES
PLANT PART USED	FRUIT	COMPLIES
SOLUBILITY	SOLUBLE (IN H_2O), SOLUBLE (IN C_2H_5OH)	COMPLIES
CHEMICAL TESTS:		
ANTHOCYANIDINS(UV)	≥25%	25.60%
EXTRACT SOLVENTS	ETHANOL & WATER	COMPLIES
SOLVENTS RESIDUE	<0.05%	COMPLIES
CARRIERS USED	NONE	COMPLIES
LOSS ON DRYING	< 5.0%	3.20%
ASH	< 5.0%	1.09%
HEAVY METALS	< 10PPM	COMPLIES
ARSENIC (As)	< 0.5PPM	COMPLIES
LEAD (Pb)	< 0.5PPM	COMPLIES
CADMIUM (Cd)	< 0.05PPM	COMPLIES
MERCURY (Hg)	NOT DETECTED	COMPLIES
PESTICIDE RESIDUE (GC)		
666	<0.1 PPM	COMPLIES
DDT	<0.1 PPM	COMPLIES
ACEPHATE	<0.1 PPM	COMPLIES
METHAMIDOPHOS	<0.1 PPM	COMPLIES
PARATHION	<0.1 PPM	COMPLIES
PCNB	<10 PPB	COMPLIES
MICROBIOLOGICAL TEST		
TOTAL PLATE COUNT	<1000CFU/G	COMPLIES
YEAST AND MOLD	<100 CFU/G	COMPLIES
SALMONELLA	NEGATIVE	COMPLIES
E.COLI	NEGATIVE	COMPLIES
STAPHYLOCOCCUS	NEGATIVE	COMPLIES
AFLATOXINS	<0.2 PPB	COMPLIES
EXTRACT METHOD	ETHANOL & WATER EXTRACTION AND SPRAY DRY.	
STORAGE	STORE IN COOL AND DRY PLACE. KEEP AWAY FROM STRONG LIGHT AND HEAT.	
PACKING	20KG/DRUM I.D.42CM × H52CM	

QC_____　　QA_____

Analysis Method of Anthocyanidins in European Bilberry Extract (UV)

1 Equipment

Electronic balance

Pipet, Volumetric flasks

Ultrasonic bath

Spectrophotometer

1cm quartz cell

2 Process

To about 10mg(W), accurately weighed, add 20ml of 2% hydrochloric acid Methanol solution and ultrasonic bath until sample has dissolved, Cool to room temperature, then diluting to volume with 2% hydrochloric acid Methanol solution to 50ml (L1) exactly. Then take 5ml(L2) of this solution , dilute with 2% hydrochloric acid Methanol solution to 50ml exactly.

3 Assay

Measure the extinction (A) of the solution at the maximum at about 540nm in 1cm cell, using 2% hydrochloric acid Methanol solution as the blank.

4 Calculation

$$\text{Content of ANTHOCYANIDINS (\%)} = \frac{A \times 50ml \times 0.5}{M\,(1-m)\ 5ml \times 1020} \times 100\%$$

1020: coefficient of Delphinidin

M: Weight of sample (g)

m: content of moisture in sample

A: Absorbance of the sample solution