Hepatocyte Cytotoxicity Induced by Hydroperoxide (Oxidative Stress Model) or Dicarbonyls (Carbonylation Model): Prevention by Bioactive Nut Extracts or Catechins

By

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Abstract

Hepatocyte Cytotoxicity Induced by Hydroperoxide (Oxidative Stress Model) or Dicarbonyls (Carbonylation Model): Prevention by Bioactive Nut Extracts or Catechins

Masters of Science, 2009

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Carbonyl and oxidative stress augment the development of diabetic complications. We evaluated the cytoprotectiveness of walnut and hazelnut extracts and catechins for decreasing cytotoxicity, lipid peroxidation, reactive oxygen species (ROS) formation, and protein carbonylation in cell death models of carbonyl and oxidative stress. Polar extracts (methanol or water) showed better cytoprotection than the non-polar (ethyl acetate) nut extracts against hydroperoxide-induced hepatocyte cell death and oxidative stress markers. Catechin flavonoids found in plants, including walnuts and hazelnuts, prevented serum albumin carbonylation in a carbonyl stress model (using glyoxal or methylglyoxal). Hepatocyte protein carbonylation and cell death were prevented and UV spectra data suggested a catechin:methylglyoxal adduct was formed. We conclude that (a) bioactive nut constituents in polar extracts were more protective than non-polar extracts against oxidative stress, and (b) catechins were effective under physiological temperature and pH, at preventing dicarbonyl induced cytotoxicity likely by trapping dicarbonyls or reversing early stage carbonylation.

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Jenkins DJ, Kendall CW, McKeown-Eyssen G, Josse RG, Silverberg J, Booth GL, Vidgen E, Josse AR, Nguyen TH, Corrigan S, **Banach MS**, Ares S, Mitchell S, Emam A, Augustin LS, Parker TL, & Leiter LA. Effect of a low-glycemic index or a high-cereal fiber diet on type 2 diabetes: a randomized trial. *JAMA*. 300(23), 2742-2753 (2008).

Wong JMW, Josse AR, Augustin L, Esfahani A, **Banach MS**, Kendall WC, & Jenkins DJ. "Glycemic Index and Glycemic Load: Effects on Glucose, Insulin and Lipid Regulation" in *Nutraceuticals, Glycemic Health and Type 2 Diabetes*. Edited by PasupuletiVK and Anderson JW. Blackwell Publishing (2008).

Abstracts:

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List of Abbreviations

AAPH	2,2'-Azobis(2-methylpropionamidine) dihydrochloride
AG	Aminoguanidine
AGE	Advanced glycation endproduct (s)
ALE	Advanced lipoxidation endproduct (s)
ANOVA	Analysis of variance
ATP	Adenosine-5-triphosphate
BHA	Butylated hydroxyanisole
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
CARE	Clinical Assessment of the Reliability of the Examination
CG	Catechin gallate
CHD	Coronary heart disease
CML	N^{ε}-(carboxymethyl)lysine
CO_2	Carbon dioxide
CVD	Cardiovascular disease
DCFH-DA	2′,7′-dichlorofluorescin diacetate
DCF	Dichlorofluorescein
DCFH	Dichlorofluorescin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl
DNPH	2,4-dinitrophenylhydrazine
DPPH	1,1-diphenyl-2-picrylhydrazyl radicals
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ETC	Electron transport chain
FDA	Food and Drug Administration
Fe	Iron
FRAP	Ferric-reducing ability of plasma
GC	Gas chromatography
GK	Goto-Kakizaki
GLO I	Glyoxalase I
GO	Glyoxal
GPx	Gluthathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen Peroxide
HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LDL	Low-density lipoprotein
MDA	Malondialdehyde

MGO	Methylglyoxal
MMP	Mitochondrial membrane potential
MPT	Mitochondrial potential transition
MS	Mass spectroscopy
MUFA	Monounsaturated fatty acid
MW	Molecular weight
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NO	Nitric oxide
O_2	Oxygen
PUFA	Polyunsaturated fatty acid
P450s	Cytochrome P450(s)
RCS	Reactive carbonyl species
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPM	Revolutions per minute
SE	Standard error
SFA	Saturated fatty acid
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TbuOOH	Tertiary butyl hydroperoxide
TCA	Tricholoroacetic acid
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy
TG	Triglycerides
TPTZ	2,4,6-Tri(2-pyridyl)-s-triazine
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UV	Ultraviolet

Chapter 1 – Introduction/Background

Oxidative Stress and Chronic Disease

The oxidation of macromolecules (proteins, lipids, and nucleic acids) is thought to be involved in the development and pathogenesis of chronic diseases (such as cardiovascular disease, type 2 diabetes, cancer and Alzheimer's disease) and in the process of aging. Normal levels of oxidation occur in the body as part of metabolic processes, and are balanced by endogenous antioxidant systems and by exogenous antioxidants (Halliwell 1994; Morrell 2008). For example, some metabolic processes which require oxygen radicals include the immune system function (i.e. the attack on pathogens by activated macrophages); and the regulation of platelet activation, signal transduction and the inhibition of endothelial cell stimulation, all of which are mediated by nitric oxide (NO) (Morrell 2008).

Oxygen radicals are highly reactive and unstable and result in oxidative damage to cells when not quenched by antioxidants (Halliwell 1996). Therefore, the term 'oxidative stress' refers to when the scavenging effects of antioxidants is outweighed by the production of free radicals. Over time, the resulting elevated oxidative stress damages essential proteins, and consequently, the functionality of proteins and DNA decreases or is changed (Halliwell 1994). Current research strongly suggests oxidative stress as a key perpetrator in the tissues which are adversely affected by several chronic diseases (Singal et al. 1998; Finkel et al. 2000; Baynes et al. 1999). Other evidence suggests that oxidative stress is not only involved in cellular damage, but may also be

involved in the upregulation or downregulation of various genes, thus altering signalling pathways, and the expression of biologically important proteins (Fang et al. 2002).

Type 2 diabetes

Type 2 diabetes is a chronic metabolic disease, and is the most common form of diabetes. This disorder occurs as a result of insulin resistance, whereby insulin loses its ability to cause cellular glucose uptake. Elevated plasma glucose (hyperglycemia) results and when left uncontrolled over time, leads to the formation and accumulation of advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) believed to cause the long term complications of diabetes such as cardiovascular disease, retinopathy, neuropathy, and nephropathy (Brownlee 2001). AGEs are compounds derived from the non-enzymatic reaction of glucose with amino groups of proteins, and also lipids and nucleic acids (Peppa et al. 2003), and ALEs arise when proteins are modified by lipid peroxidation products (e.g. oxidation of PUFAs) (Miyata et al. 2000). Chronic levels of glucose may also give rise to glucose oxidation to form dicarbonyls (Brownlee 2001). An increase in the prevalence of type 2 diabetes globally is thought to be due to changes in lifestyle leading to higher rates of overweight and obese individuals (Hu 2003). These lifestyle changes have been noted as larger portion sizes and unbalanced diets, consisting of higher fat, carbohydrate and refined sugar content, as well as a lack of exercise (Shulze et al. 2005).

The mechanisms involved in the development of diabetic complications are the subject of much current research. Diabetes has been associated with increased levels of ROS along with a decreased ability to prevent oxidative stress (Rosca et al. 2002; Baynes

1991; Baynes 1999). It is hypothesized that chronic hyperglycemia leads to a cascade of events via several pathways, ultimately forming reactive oxygen species (ROS) and reactive carbonyl species (RCS), both of which have been implicated as possible causative factors for micro- and macro-vascular tissue damage, and the development of long-term complications in type 2 diabetes (Brownlee 2001; Sheetz 2002; Beisswenger 2003). More recently, it has been hypothesized that fluctuations in blood glucose levels may be more detrimental in diabetes because of the overproduction of ROS (superoxide anions) through the mitochondrial electron transport chain, than chronic hyperglycemia (Brownlee et al. 2006; Hirsch et al. 2005). In one particular study, acute hyperglycemia caused an imbalance of the cellular redox state in humans leading to an oxidative stress situation which suggests that repeated postprandial hyperglycemia augments the cellular redox state, and over time, may lead to the development of diabetes and its complications (Miyazaki et al. 2007). Additionally, another study has shown that isoprostane output in type 2 diabetic subjects was related to glycemic fluctuations in diabetes, as isoprostanes reflect oxidative stress (specifically, the free radical-mediated oxidation of arachidonic acid) (Monnier et al. 2006). Therefore, it is hypothesized that acute hyperglycemia causes an overload of the metabolic pathways involved in the detoxification of ROS and RCS. The key perpetrator in causing the increased ROS formation is directed to the involvement of mitochondria. Specifically, hyperglycemia is thought to lead to an excess production of superoxide radical production through the mitochondrial electron transport chain (ETC). This was evident from *in vitro* studies where endothelial cells were depleted of mitochondrial DNA and lacked the functional mitochondrial ETC, which consequently resulted in a lack of ROS production from hyperglycemia (Brownlee 2005). The

imbalance of reduction and oxidation reactions *in vivo* is evident in humans, as markers of oxidative stress are known to be elevated in diabetic patients (Kesavulu 2001; Abou-Seif 2004; Pennathur 2004).

Glycemic control in type 2 diabetic patients includes drug therapy (use of oral hypoglycemic agents) as well as regular exercise, weight loss and a balanced diet (Bolen 2007; Krentz 2005; Franz 2002; Diabetes Care 2008); however, oral hypoglycemic agents may cause side effects (Lebovitz 2002; Krentz 1994) and therefore, non-pharmacological strategies to reduce or prevent the onset diabetic complications are of interest. Many foods, most notably fruits, vegetables, nuts and seeds are good sources of antioxidants and other bioactive constituents (Halvorsen 2002; Pellegrini 2006). Therefore, studying dietary sources of antioxidants may be useful in the context of ameliorating oxidative stress seen in type 2 diabetes and other chronic diseases.

The Maillard (Browning) Reaction and Carbonyl Stress

The Maillard reaction is a non-enzymatic browning reaction, named after the physician and chemist Louise-Camille Maillard who studied this reaction in the early 1900s while studying food chemistry (Henle et al. 1996; Gugliucci 2000). Maillard discovered that sugars reacted with amino acids of a wide range of proteins, in a non-enzymatic glycation reaction, ultimately yielding low molecular weight (MW) heterocyclic flavour compounds or high MW brown pigments (Henle et al. 1996). The non-enzymatic reaction occurs between amino acids and reducing sugars. Specifically, the reactive carbonyl groups of sugars react with the nucleophilic amino groups of amino acids which autoxidize to form AGEs (Figures 1-1, 1-2). The Maillard reaction has

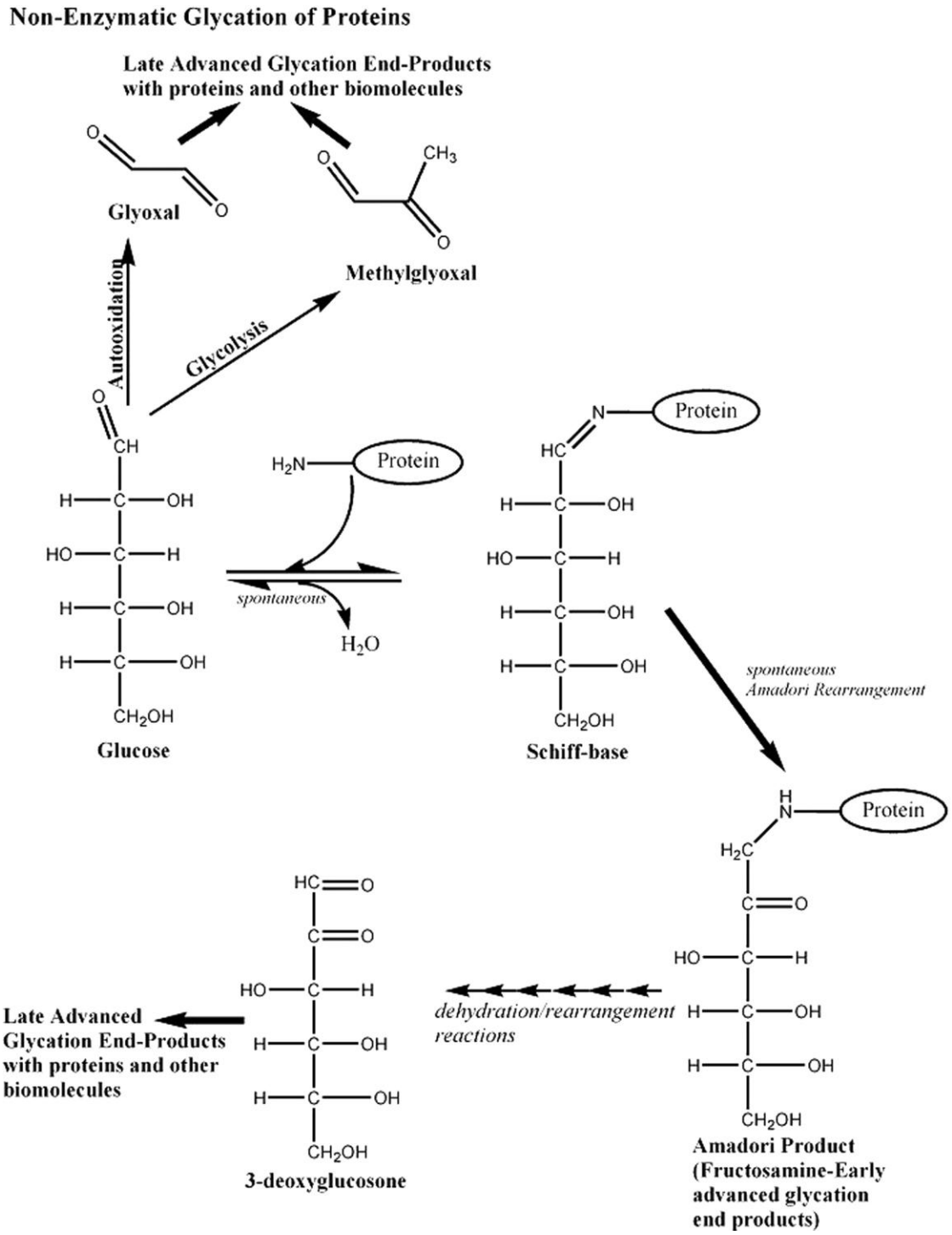


Figure 1-1: Non-enzymatic Glycation. Non-enzymatic glycation initially begins with glucose reacting with the amino group of an amino acid of a protein (i.e. lysine or arginine), with intermediates formed (Schiff Base adduct; Amadori Product), ultimately leading to the formation of more stable advanced glycation end products (AGEs) (O'Brien et al. 2005).

been noted to occur in the preparation of many foods, and more recently, has been hypothesized to be involved in the endogenous formation of irreversible protein modifications which are known as protein carbonyls (Njoroge et al. 1989; Vlassara et al. 2002). The term 'carbonyl stress' describes elevated levels of protein carbonyls compared to endogenous protein carbonyl levels found in the body. In diabetes, elevated glucose levels were found to induce protein glycation and glucose autoxidation (Baynes et al. 1999; McCance et al. 1993; Dyer et al. 1993), eventually leading to the formation of irreversible protein modifications (protein carbonyls) known as advanced glycation end products (AGEs) or advanced lipoxidation end products (ALEs), through the Maillard reaction (Sheetz 2002; Gugliucci 2000). Prior to AGE formation in the Maillard reaction, early stage reversible intermediates exist and are known as Schiff base adducts and Amadori products. Research on therapeutic agents to prevent these chemical modifications of proteins have largely focused on the early-stage prevention of AGE precursors, such as reversing Schiff Base adducts or inhibiting Amadori product formation by preventing both oxidative and carbonyl stresses (Sullivan 2005; Rahbar 2003). In the Maillard reaction, highly reactive α-oxo aldehydes or dicarbonyls such as methylglyoxal (MGO) and glyoxal (GO), are much more effective than sugars at forming AGE or advanced lipoxidation end products (ALEs) or their precursors (Thornalley 1999; Aldini 2007; Vlassara et al. 2002). MGO is largely formed from the triose phosphate glycolytic intermediates of glucose or fructose metabolism (Beisswenger 2003; Rosca 2002) whereas GO on the other hand, is formed during autoxidation or Fenton's catalyzed oxidation of glucose or fructose or their metabolites (Thornalley 1999; Aldini

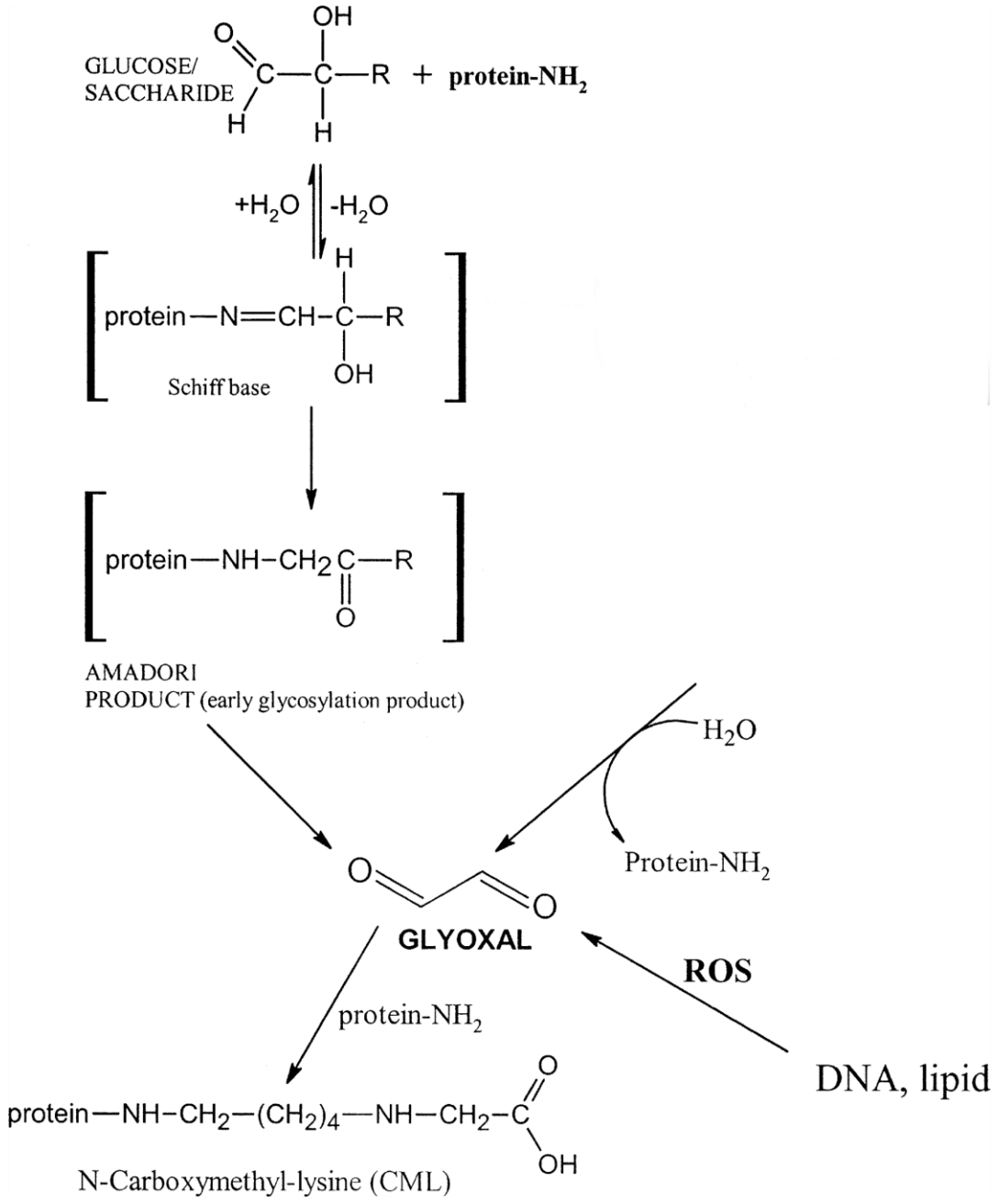


Figure 1-2: Glycation through the Maillard reaction. Endogenous glyoxal formation by autoxidation of glycoaldehyde through the Maillard reaction, occurring between a reducing sugar and an amino group of proteins (known as glycation), which results in the conversion of reversible Schiff base (imine) adducts to covalently bound Amadori rearrangement products (a reversible step). Amadori products can then undergo multiple dehydration and rearrangement reactions resulting ultimately in AGE products or producing glyoxal. Glyoxal can react with one lysine to form the oxidative adduct CML, an oxidative AGE product (O'Brien et al. 2005).

2007; O'Brien et al. 2005). Plasma GO and MGO levels have been measured and found to be elevated several fold in diabetics (Lapolla et al. 2003; Thornalley 1999; Han 2007; Beisswenger 1999) and several AGE products have been identified (O'Brien et al. 2005; Baynes et al. 1999; Ahmed et al. 2007). Also, these dicarbonyls react readily with proteins; specifically, lysine and arginine residues are binding sites for dicarbonyls, ultimately leading to the formation of AGEs (Ahmed & Thornalley 2007).

Free Radicals

Free radicals are highly reactive and unstable molecules which have an unpaired electron in the outer orbit, and actively search to gain an electron (Fang et al. 2002) in order to have paired electrons. Free radicals containing oxygen are known as reactive oxygen species (ROS) or whilst those also containing nitrogen are known as reactive

Table 1-1: Free radicals and related non-radical oxidizers

ROS[i]	Free radicals	alkoxyl ($RO\cdot$), hydroperoxyl ($HO_2^{\cdot}$) hydroxyl ($OH\cdot$), peroxyl ($RO_2^{\cdot}$), superoxide ($O_2^{\cdot-}$)
	Non-radical oxidizers	hydrogen peroxide (H_20_2), hypochlorous acid (HOCl), ozone (O_3)
RNS[ii]	Free radicals	nitric oxide ($NO\cdot$), nitrogen dioxide ($NO_2^{\cdot}$)
	Non-radical oxidizers	alkyl peroxynitrites (ROONO), dinitrogen tetroxide (N_2O_4), dinitrogen trioxide (N_2O_3), nitrogen dioxide (NO_2), nitronium cation (NO_2^{+}), nitrous acid (HNO_2), peroxynitrite ($ONOO^{-}$), peroxynitrous acid (ONOOH),

[i]Reactive oxygen species (ROS) describes both radical and non-radical species and the non-radical species act as oxidizers and/or may easily transform into radical species. Similarly,
[ii]Reactive nitrogen species (RNS) describes both radical and non-radical species and the non-radical species act as oxidizers and/or may easily transform into radical species.
(adapted from Halliwell 1996)

nitrogen species (RNS). ROS and RNS may also give rise to non-reactive species as well. Over time, the accumulation of damaged macromolecules caused by free radicals potentially leads to altered or decreased functioning of physiologically relevant molecules, and results in tissue damage, altered or damaged nucleic acids, or disease (Fang et al. 2002).

Antioxidants

Antioxidants function to prevent the formation of free radicals and to scavenge free radicals. The human body has several means of providing an antioxidant defence which include:

1) antioxidant enzyme systems (i.e. superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx)) which act to remove free radical species by transforming them into less toxic or non-toxic molecules;

2) endogenous antioxidants (i.e. glutathione (GSH));

3) exogenous antioxidants obtained through the diet (i.e. vitamin C, carotenoids) (Halliwell 1996; Fang et al. 2002).

Antioxidants may be either water soluble or lipid soluble. Therefore, because antioxidants partition into different physiological membranes, depending on their solubility and access to the area, they may act on different biological components (i.e. cytosol localization for water soluble antioxidants versus membrane localization for lipid soluble antioxidants) (Fang et al. 2002).

Antioxidants have been studied for their favourable health effects such as lowered risk in cancers, diabetes, and coronary heart disease (CHD). Lowered risks in these

chronic diseases have been associated with increased consumption of antioxidant rich foods. Many fruits, vegetables, nuts and seeds have been evaluated for their antioxidant content to determine their antioxidant potential or reducing ability of free radicals. Interestingly, water soluble compounds contributed to > 90% of the total antioxidant potential of most foods (Wu et al. 2004). Different varieties of antioxidants from dietary sources exist and include a wide range of compounds such as vitamins (i.e. vitamin C and E), carotenoids (i.e. β-carotene), and polyphenolic compounds (i.e. flavonoids, resveratrol).

Composition of Commonly Consumed Nuts

Nuts are both seed and fruit, produced by some plant varieties, although peanuts (groundnuts) are not true nuts, but are legumes (Rosengarten 2004). Nuts contain a range of compounds such as unsaturated fatty acids (mono-unsaturated fats, MUFAs and poly-unsaturated fats, PUFAs), vegetable protein, plant sterols (phytosterols), fiber, micronutrients, such as vitamins, and multiple bioactive compounds (Table 2) (Kris-Etherton et al. 1999; King et al. 2008). Nuts contain relatively high levels of both water soluble and lipid soluble antioxidants (King et al. 2008; Wu et al. 2004). Studies have shown that a large proportion of the antioxidant capacity in nuts is found in the pellicle (skin) of the nut (Blomhoff 2006, Chen 2008, Wijeratne et al. 2006), while vitamin E (tocopherols) is found in the oil isolated from nut kernels, as tocopherols are lipid soluble compounds (Traber and Atkinson 2007). Traditionally, the vitamin E content of nuts and nut oils has been studied and thought to contribute to the antioxidant effect of nuts, while

Table 1-2: Macronutrients and Vitamins in Commonly Consumed Nuts*

Nutrients**	**Almond** *(Prunus Dulcis)*	**Brazil nut** *(Bertholletia excelsa)*	**Cashew** *(Anacardium occidentalle)*	**Walnut** *(Juglans Regia)*	**Hazelnut** *(Corylus avellana)*	**Macadamia nut** *(M.integrifolia or tetraphylla)*	**Peanut** *(Arachis hypogaea)*	**Pecan** *(Carya illinoinen-ses)*	**Pistachio** *(Pistacia vera)*
Energy (kcal)	575	656	553	654	628	718	567	691	557
Protein (g)	21.2	14.3	18.2	15.2	15.0	7.9	25.8	9.2	20.6
Fat (g)	49.4	66.4	43.9	65.2	60.8	75.8	49.2	72.0	44.4
SFAs (g)	3.7	15.1	7.8	6.1	4.5	12.1	6.8	6.2	5.4
MUFAs (g)	30.89	24.55	23.80	8.93	45.65	58.88	24.43	40.80	23.32
PUFAs (g)	12.1	20.6	7.8	47.2	7.9	1.5	15.6	21.6	13.5
Carbohydrate (g)	21.7	12.3	30.2	13.7	16.7	13.8	16.1	13.9	28.0
Fiber (g)	12.2	7.5	3.3	6.7	9.7	8.6	8.5	9.6	10.3
Sugars (g)	3.89	2.33	5.91	2.61	4.34	4.57	3.97	3.97	7.64
α-tocopherol (mg)	26.22	5.73	0.9	0.7	15.03	0.54	8.33	1.4	2.3
β-tocopherol (mg)	0.29	0	0.03	0.15	0.33	0	n/a	0.39	0
γ-tocopherol (mg)	0.65	7.87	5.31	20.83	0	0	n/a	24.44	22.6
Δ-tocopherol (mg)	0.05	0.77	0.36	1.89	0	0	n/a	0.47	0.8
Selenium (mcg)	2.5	1917	19.9	4.9	2.4	3.6	7.2	10	7
Vitamin C (mg)	0	0.7	0.5	1.3	6.3	1.2	0	1.1	5
Thiamin (mg)	0.21	0.62	0.42	0.34	0.64	1.20	0.64	0.66	0.87
Folate (mcg)	50	22	25	98	113	11	240	22	51
Arginine (g)	2.45	2.15	2.12	2.28	2.21	1.40	3.09	1.18	2.03

* estimates are based on United States Department of Agriculture (USDA) National Nutrient Database standard references

** values are given per 100 grams

All approximations are based on different numbers of data points

Kcal – kilocalories; g – grams; mg – milligrams; mcg - micrograms

more recent studies have investigated the water soluble antioxidants and flavonoids found in nuts.

Multiple factors may affect the composition of nuts such as environmental differences in climate, soil, cultivars, as well as cooking practices, storage conditions, and differences in varieties of nuts (Lavedrine et al. 1997).

Epidemiological Studies Showing Health Effects of Nuts

Several large prospective cohort studies (the Adventist Health Study, the Iowa Women's Health Study, the Nurses' Health Study, the Physicians' Health Study, and the CARE Study) suggested that frequent nut consumption may have a protective effect against coronary heart disease (CHD) as all studies found an inverse association between nut consumption and CHD risk (Hu and Stampfer 1999). Hu et al. recommended regular nut consumption and estimated that an exchange of 1 ounce of nuts for energy in carbohydrates was associated with a 30 % risk reduction of CHD (Hu and Stampfer 1999). Nut consumption has also been associated with a reduced risk of developing type 2 diabetes in women (Jiang et al. 2002). In 2003, the Food and Drug Administration (FDA) issued a new health claim for tree nuts, stating that consumption of 1.5 ounces of nuts may reduce the risk of heart disease (Sabate et al. 2006). A beneficial effect of nuts on blood lipid profiles in humans was examined in a systematic review, which found that a consumption of nuts (in particular, almonds, walnuts, pecans and peanuts) of 1.5 – 3.5 servings ≥ 5 times per week was associated with a significantly lower total cholesterol and low-density lipoprotein (LDL) cholesterol in healthy and hyperlipidemic subjects (Mukuddem-Peterson et al. 2005).

Table 1-3: Flavonoids and Polyphenolic Compounds identified in Nuts

Nut	Flavonoids and/or Polyphenols	Reference
Almond	EGC, cyanidin, GCG, EC, C Isorhamnetin 3-*O*-glucoside, Isorhamnetin 3-*O*-rutinoside, C, kaempferol 3-o-rutinoside, EC, quercetin 3-*O*-galactoside, Isorhamnetin 3-*O*-galactoside	Harnley et al. 2006 Milbury et al. 2006
Brazil nut	None detected	King et al. 2008
Cashew	EC, EGC	Harnley et al. 2006
Hazelnut	cyanidin, EGC, C, EGCG, GCG, EC; gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid gallic acid, p-hydroxybenzoic acid, EC, caffeic acid, sinapic acid and quercetin	Harnley et al. 2006 Shahidi et al. 2007 Yurttas et al. 2000
Macadamia	None detected	King et al. 2008
Peanut	Resveratrol Dihydroquercetin (Spanish peanut)	Sanders et al. 2000 Pratt et al. 1984
Pecan	Cyanidin, delphinidin, C, EGC, EGCG, EC, GCG	Harnley et al. 2006
Pistachio	Cyanidin, C, EGC, quercetin, EC, GCG	Harnley et al. 2006
Walnuts	Gallic acid, C, caffeic acid, coumaric acid, ellagic acid Ellagic acid, valoneic acid dilactone (Persian walnut) Ellagitannins, glansrins A, B and C	Papoutsi et al. 2008 Li et al. 2006 Fukuda et al. 2003

C – catechin; EC – epicatechin; EGC – epigallocatechin; EGCG – epigallocatechin gallate; GCG – gallocatechin gallate.

Bioactive compounds in Nuts

The antioxidant potential of nuts in chemical assays indicates that nuts contain relatively high levels of antioxidants such as α-tocopherol, γ-tocopherol, caffeic acid, ellagic acid, *p*-coumaric acid, gallic acid, epicatechin, and quercetin (Halvorsen et al. 2002, Pellegrini et al. 2006, Chen et al. 2005, Shahidi et al. 2007).

By virtue of their bioactive constituents and antioxidant content, tree nuts provide a potential dietary method of reducing oxidative stress and/or elevating plasma antioxidants (Kris-Etherton et al. 1999). Kris-Etherton has suggested that components, other than the unsaturated fatty acid profile of nuts, contributed to the cholesterol-

Table 1-4: Evidence of Beneficial Health Effects of Nuts in Clinical Human Trials

Nut	Health Benefits Associated with Nut	Trial Design and Subjects	Length of Trial	References
Almond	(i) ↓ postprandial glycemia, insulinemia and oxidative damage.	Several single diet interventions (healthy subjects)	5 sessions of 4 hours each (1 week washout periods)	Jenkins et al. 2006
	(ii) Improved plasma α-tocopherol and ↓ plasma lipids	Randomized crossover feeding trial (dose response in healthy subjects)	3 consecutive 4 week cycles	Jambazian et al. 2005
Brazil nut	↑ selenium status, increased GPx activity	Randomized controlled trial (healthy subjects)	12 weeks	Thomson et al. 2008
Cashew	Improvements in baroreflex sensitivity	Randomized parallel controlled design (subjects with metabolic syndrome)	8 weeks	Schutte et al. 2006
Hazelnut	Enhanced plasma antioxidant potential, ↓ plasma cholesterol	Single intervention (healthy subjects)	30 days	Durak et al. 1999
Macadamia	↓ total and LDL cholesterol	Randomized crossover controlled design (subjects with mild hypercholesterolemia)	5 week periods for each diet	Griel et al. 2008
Peanut	↓ total cholesterol and triglycerides (TG)	Randomized crossover trial (healthy subjects)	30 weeks	Lokko et al. 2007
Pecan	↓ total and LDL cholesterol and TG, and ↑ HDL cholesterol	Single-blind randomized controlled crossover feeding trial (healthy subjects)	4 weeks per diet	Rajaram et al. 2001
Pistachio	(i) ↓ oxidative stress; and improved total and HDL cholesterol	Randomized controlled trial (healthy subjects)	3 weeks	Kocygit et al. 2006
	(ii) ↓ in ratios (TC/HDL; LDL/HDL; B-100/A1); ↑ HDL	Randomized crossover (mild hypercholesterolemic subjects)	4 weeks	Sheridan et al. 2007
	(iii) Improved CVD risk factors	Randomized crossover (elevated LDL subjects)	4 weeks for 3 separate diets	Gebauer et al. 2008
Walnut	(i) Improved lipid profile (↑ HDL:Total cholesterol ratio; ↑ HDL; ↓ LDL)	Parallel randomized controlled trial (type 2 diabetic subjects)	6 months	Tapsell et al. 2004
	(ii) ↓ plasma TG and ↑ plasma HDL	Randomized case controlled study (subjects with elevated TG or total cholesterol)	8 weeks	Zibaeenezhad et al. 2005
	(iii) ↓ in LDL, HDL, total cholesterol, and LDL:HDL ratio	Controlled, single-blind, randomized, cross-over (healthy male subjects)	4 weeks for each diet	Sabate et al. 1993

lowering effect of nuts, as the cholesterol lowering response was much greater than what had been predicted (Kris-Etherton et al. 1999).

Nut consumption in human clinical trials

The antioxidant and lipid lowering effects of nuts have been studied in some human clinical trials, and nuts have been shown to decrease biomarkers of oxidative stress (such as oxidized LDL cholesterol) and to improve the blood lipid profile (Blomhoff 2006).

Several clinical studies have indicated that consumption of tree nuts lowers serum lipid and cholesterol levels and decreases oxidative stress in humans (Jenkins et al. 2002; Jenkins et al. 2006 Durak et al. 1999; Garg et al. 2003; Morgan et al. 2000). One study reviewed 18 clinical feeding trials and found that when nuts were included in the subjects' test diets, there was a 25 % decrease in cholesterol and the additional cholesterol lowering effect was attributed to other non fatty acid constituents of nuts (Kris-Etherton 1999). Table 4 lists the health benefits associated with consumption of different nuts, noted in human clinical trials.

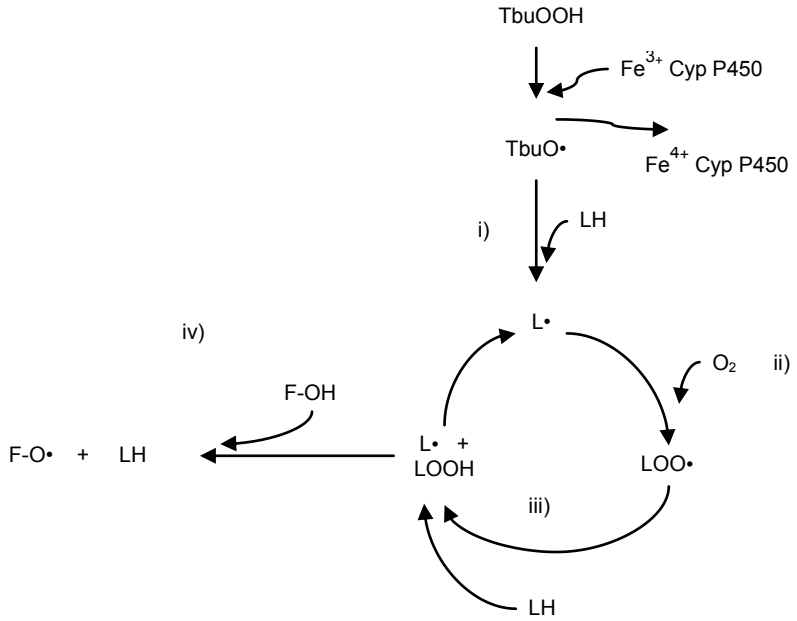
In Vitro Experiments using Nuts and Nut Extracts

Few *in vitro* studies have investigated the direct effect of nuts in biological assays. Most *in vitro* studies have focused largely on lipophilic extracts of nuts or nut oils and analyses determined that the composition consisted mainly of lipid soluble antioxidants such as α-tocopherol, γ-tocopherol and tocotrienols (Crews et al. 2005; Crews et al. 2005; Maguire et al. 2004; Li et al. 2007; Alasalvar et al. 2003). More recently, the total

antioxidant activity (TAC) of the polyphenolic hydrophilic extract of Sicilian pistachio nuts was found to be 50 fold higher compared to the antioxidant activity of the tocopherol containing lipophilic extract (Gentile et al. 2007), suggesting that more polar (water soluble) compounds were more effective than non-polar antioxidants.

Mechanisms of Bioactive Compounds in Nuts against Oxidative Stress

Due to the variety of polyphenolic compounds found in nuts, it is likely that multiple protective mechanisms may act against oxidative and carbonyl induced cytotoxicity in *in vitro* models. One possible mechanism whereby nuts may decrease oxidative stress in cells is in the prevention of lipid peroxidation. Figure 1-3 outlines the



i) LPO initiation
ii) LPO propagation
iii) Further LPO propagation
iv) Antioxidant activity of phenolic flavonoids through scavenging the peroxy radical

Figure 1-3: The Lipid Peroxidation Cycle and the Antioxidant Mechanism of Phenolic Flavonoids against Lipid Peroxides. (TbuOOH - tertiary butyl-hydroperoxide; TbuO· - tert-butyl alkoxyl radical; LH – lipid; L· - lipid radical; LOO· - lipid peroxy radical; F-OH – flavonoid)

mechanism by which an organic hydroperoxide (tertiary butyl hydroperoxide, TbuOOH) induces lipid peroxidation (LPO), and the antioxidant mechanism by which polyphenolics act to prevent the LPO cycle. Polyphenolic flavonoids abstract an electron from the lipid centre radical (L•), and form a more stable radical (F-O•), which is then reduced by other flavonoids or reductants.

Importantly, synergistic effects between antioxidants could exist in which antioxidants recharge neighbouring antioxidants (Blomhoff et al. 2006). For example, flavonoids from almond skins were found to act synergistically and were more effective with vitamin C or vitamin E, compared to the almond skins or vitamins alone, to protect against LDL oxidation *in vitro* as well as enhancing resistance to Cu^{2+} induced oxidation of LDL *ex vivo* (Chen et al. 2005). Another study found that quercetin and epicatechin regenerated α-tocopherol thereby eliciting a co-antioxidant effect (Pedrielli et al. 2002). The superoxide radical scavenging ability of the polyphenolics in ethanolic almond nut extracts is also a likely contributor to the antioxidative properties of almond extracts, and possibly other nuts containing polyphenolics (Siriwardhana 2002). Also, almond extracts demonstrated excellent metal (iron) chelating abilities, which was attributed to phenolic compounds (Wijeratne et al. 2006).

Catechins

Catechins are polyphenolic plant metabolites which belong to a subset of flavonoids, known as flavan-3-ols (Figure 1-4). Catechins are characterized by a 2-phenyl-3,4-dihydro-2H-chromen-3-ol skeleton and are predominantly found in teas

(Lachman et al. 2009; Aron and Kennedy 2008). However, these compounds have also been found in a variety of plants and fruits, including nuts (Aron and Kennedy 2008).

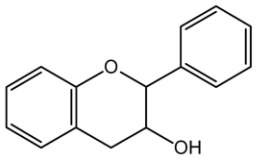
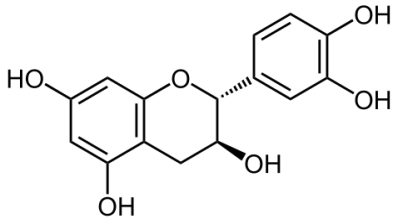
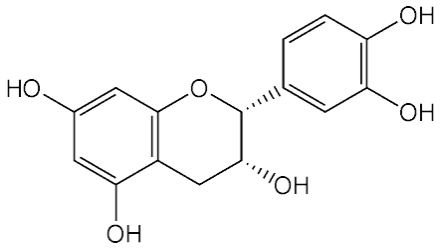


Figure 1-4: Structure of Flavan-3-ol

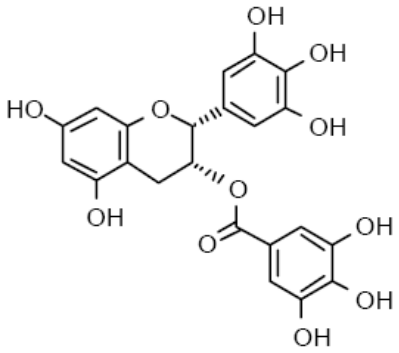
Common catechins are catechin, epicatechin (an epimer of catechin), gallocatechin, epigallocatechin (EGC), epicatechin gallate (ECG), catechin gallate (CG), and epigallocatechin gallate (EGCG). Research has indicated that catechins are linked to



(+)-catechin



(-)-epicatechin



(-)-epigallocatechin gallate (EGCG)

Figure 1-5: Structures of Catechin, Epicatechin and EGCG

several beneficial health effects, and act as antioxidants. For example, due to their very high content of catechin compounds, especially EGCG, teas have attracted much attention for their beneficial protective effects against cardiovascular disease and cancer (Cabrera et al. 2006). Sano et al. noted that green tea consumption was associated with a lower incidence of coronary arterial disease among Japanese populations (Sano et al. 2004). Strong evidence of chemoprotective abilities of green tea catechins and EGCG has been demonstrated in several animal models (Chung et al. 2003). Other noted beneficial health effects by green tea catechins have been shown for type 2 diabetes, neurodegenerative diseases, obesity, weight loss, and microbial diseases (Zaveri 2006). Despite the wide array of publications on the health outcomes of catechin, the unanswered question still remains as to the bioavailability of catechins and ultimately, the preventative mechanisms occurring *in vivo* in humans. Nonetheless, more studies of clinical trials in humans would aid to further understand the bioavailability and metabolism of these compounds.

In the food industry, amino acids and other compounds have been investigated for their binding capacities with carbonyl containing aldehydes, which form colourful adducts, that are responsible for aromas and flavours of wines and certain foods (Marchand et al. 2000). Marchand et al. demonstrated that the amino acid cysteine reacted with different dicarbonyls in model solutions of wine conditions, to produce aromatic compounds (Marchand et al. 2000). Another study found that catechin formed an adduct with acetaldehyde or glyoxylic acid or with both aldehydes, in a wine-like model solution (Drinkine et al. 2005).

More recently, catechins have been investigated for their binding abilities to dicarbonyls methylglyoxal (MGO) and glyoxal (GO) in an *in vitro* aqueous Maillard system (Totlani et al. 2006), and direct reactivity of compound was indicated. Additionally, two methylglyoxal (MGO)-epicatechin adducts were identified (Totlani et al. 2006). Furthermore, MGO was found to react and form adducts with several catechins under physiological conditions (pH 7.4, 37 ° C) in a phosphate buffer system (Lo et al. 2006). These observations indicate the possible role of catechins in reducing dicarbonyls by forming stable adducts, and may suggest another beneficial health effect in the prevention of carbonyl and oxidative stress by catechin-containing foods such as nuts, particularly in type 2 diabetes and possibly other chronic diseases.

Tertiary-butyl hydroperoxide as a Model for Inducing Oxidative Stress

Tertiary butyl-hydroperoxide (TbuOOH) has been used in *in vitro* cell systems to induce oxidative stress, lipid peroxidation, and cell death (Hwang 2005). This organic hydroperoxide has been used in experiments involving isolated rat hepatocytes, and the oxidative stress mechanism involves the Fenton reaction, whereby iron reacts with the hydroperoxide to produce highly reactive hydroxyl radicals, and consequently cause cellular damage (Yamashiro et al. 2008). TbuOOH has been shown to trigger both apoptosis at lower concentrations (before necrosis ensues), and necrosis in cultured hepatocytes, by increasing NADPH oxidation, which subsequently increases mitochondrial calcium (Ca^{2+}) (Gomez-Lechon et al. 2002; Lemasters et al. 1999). Mitochondrial ROS formation, in part with other cellular alterations, leads to the mitochondrial potential transition (MPT) (Gomez-Lechon et al. 2002). Apoptosis or

necrosis ensues, and the resulting path is dependent on the MTP effect on ATP levels. For example, when ATP levels decline rapidly, necrosis is the outcome, whereas, a more minimal loss of ATP leads to the apoptotic pathway (Lemasters et al. 1999). Hence, the progression to necrotic cell death in hepatocytes induced by TbuOOH is dependent on the concentration of the hydroperoxide, as a large production of ROS causes a significant effect on the mitochondrial permeability, and hence, leads to necrotic cell death. Due to the major involvement in ROS production in mediating cytotoxicity by TbuOOH, this compound has been used as a model of oxidative stress-induced cell death.

Methylglyoxal and Glyoxal as Models for Carbonyl Stress

Methylglyoxal (MGO) and glyoxal (GO) are α-oxo-aldehydes which are potent glycating agents, and have been implicated in the AGE pathway in the pathogenesis of type 2 diabetes and other chronic diseases. Both of these compounds have been implicated in glucotoxicity through the AGE pathway, and ultimately, in the mechanism of diabetic complications (Ahmed and Thornalley 2007).

Methylglyoxal Glyoxal

Figure 1-6: Structures of the α-oxo-aldehydes, Methylglyoxal and Glyoxal. Both compounds are highly reactive dicarbonyls and potent glycating agents. Methylglyoxal and glyoxal have been implicated in AGE pathway in the pathogenesis of type 2 diabetes and other chronic diseases.

In isolated rat hepatocytes, GO has been shown to induce lipid peroxidation, increase ROS formation, deplete glutathione (GSH) levels, and collapse the mitochondrial membrane potential (MMP) before causing cytotoxicity (Shangari and O'Brien 2004). MGO alone is not cytotoxic to hepatocytes. However, when hepatocytes were depleted of GSH by using low and non-toxic concentrations of 1-bromoheptane, MGO was found to be cytotoxic (Shangari et al. 2006). MGO was also cytotoxic to hepatocytes when sorbinil was used to inhibit aldose reductase (Shangari et al. 2006). It is evident that both of these dicarbonyl compounds cause cellular damage. Due to the involvement of these compounds in diabetes, thought to occur by oxidative and carbonyl stress mechanisms, both dicarbonyls are useful compounds for investigating therapeutics to prevent these cellular stresses, such as carbonyl stress.

Scope of Thesis

Despite large scale epidemiological studies which indicate a protective effect of nut consumption and a reduced risk in type 2 diabetes with increasing nut consumption, few *in vitro* studies have investigated the direct effect of nuts on cytotoxicity prevention in biological assays, and more specifically, in a model of oxidative stress. The antioxidant potential of nuts in chemical assays indicates that nuts contain relatively high levels of antioxidants. Therefore, the aim of our research was to screen different nut extracts for their cytoprotective abilities in an *in vitro* model of oxidative stress cell death. Data on nut extracts have mainly included antioxidant assays used in determining their antioxidant capacities. However, the potential effect of nut extracts on protecting against

oxidative stress in intact, living cells has not been elucidated. Also, observing the effect of different nut extracts may further indicate the protective mechanism in cellular models. Our hepatocyte model was a unique method to determine differences in cytoprotection by different nut extracts. Thus, our study strengthens the data on the ability of nut extracts (made using whole nuts with skin) to exert a protective effect by preventing cell death and other markers of oxidative stress.

The pathogenesis of diabetes also involves carbonyl stress. Catechins have been shown to form adducts with some aldehydes in chemical assays. Therefore, we investigated several catechins (epicatechin, catechin and EGCG), which are also found predominantly in teas but also in hazelnuts and walnuts, for their ability to prevent carbonyl formation in a cell free model, using bovine serum albumin (BSA) and to prevent carbonyl stress induced cell death and protein carbonylation in an *in vitro* model, using primary hepatocytes.

Chapter 2 – Research Aims, Objectives and Hypotheses

Research Aims

We wish to explore natural compounds to protect against oxidative stress and carbonyl stress, both of which are involved in the pathogenesis of type 2 diabetes as well as several other chronic diseases. Reactive oxygen species (ROS) as well as advanced glycation end product (AGE) precursors are important targets in type 2 diabetes and chronic diseases for investigating the mechanism whereby natural bioactive compounds may exert their effect.

Nuts are a good source of a wide variety of compounds, and most notably, antioxidants. Much of the literature on nuts has largely focused on nut oils and the lipid soluble antioxidant – vitamin E (tocophorols and tocotrienols). However, more recently, water soluble antioxidants have been attributed to the antioxidant capacity of most foods. The antioxidant capacity of nuts has been investigated in many studies, as well as the identification of antioxidants in nuts, however, the protection by nuts in an oxidative stress model using intact cells has not yet been elucidated. Therefore, we sought to investigate three different crude extracts of whole walnuts and hazelnuts and compare these for their ability to reduce lipid peroxidation (a marker of oxidative stress) using microsomes, and also, to investigate the cytoprotective ability of these extracts in oxidative stress-induced cell death, lipid peroxidation and ROS formation, by using tertiary butyl hydroperoxide, a known organic hydroperoxide used as an oxidant in studies using hepatocytes. Isolated hepatocytes were used as the model cell because of the relevance of the liver in intermediary metabolism of nutrients and foreign compounds

(drugs or toxins). Furthermore, the prevention of cell death by nut extracts has not yet been investigated.

Catechins, a group of flavonoids are found nuts and teas, as well as in many plant varieties. As diabetic patients not only have increased markers of oxidative stress, but also have an accumulation of carbonylated proteins, our aim was to investigate natural compounds in nuts that may possibly prevent protein carbonyl formation. Recent literature has suggested that catechins, and possibly other flavonoids, directly react with the highly reactive dicarbonyls, methylglyoxal and glyoxal (precursors and intermediates of AGE pathway), and 'trap' these dicarbonyls to form stable adducts. The effect of catechins on protein carbonylation has not yet been explored, and we therefore sought to determine the effect of catechins on dicarbonyl (methylglyoxal or glyoxal) induced protein carbonylation using a cell free model (serum albumin) and subsequently, in a cell death model (hepatocytes), also induced by dicarbonyls. The specific use of dicarbonyls to induce cytotoxicity was focused on as these compounds (methylglyoxal and glyoxal) are highly reactive and more potent glycated agents compared to glucose, and are known precursors of AGE product formation; thus, the prevention of early-stage protein carbonyl formation is a useful key target as early-stage carbonylation is reversible (e.g. Schiff base reversal).

Studying the prevention of protein carbonylation induced by methylglyoxal or glyoxal by catechins may suggest yet another protective function of nuts, whereby catechins in nuts (as well as other plants) may reduce carbonyl stress.

Objectives

a) To demonstrate that walnut and hazelnut polar extracts are more cytoprotective compared to walnut and hazelnut lipophilic extracts in an oxidative stress cell model, by assessing cytotoxicity by cell death, lipid peroxide formation, and ROS formation, as nut extracts have not been previously examined in cells.

b) To demonstrate that catechins effectively decrease dicarbonyl induced protein carbonylation formation in a cell free model (BSA) and cell model (hepatocytes), and in this way, to make evident another possible mechanism by which nuts (as well as fruits and vegetables) may reduce carbonyl stress.

Overall Hypothesis

Nut extracts will prevent oxidative stress-induced cell death. Polar extracts will elicit better cytoprotection compared to more lipophilic nut extracts. Catechin, epicatechin and epigallocatechin gallate (EGCG), found in plants and to a lesser extent in tree nuts, can prevent cell death and protein carbonylation induced by the dicarbonyls implicated in the pathogenesis of diabetes.

Specific Hypotheses

a) Walnut and hazelnut extracts prevent oxidative stress induced lipid peroxidation in liver microsomes in a dose dependent manner, where the polar extracts exert more protection than the nonpolar extracts. These nut extracts also prevent oxidative stress induced cell death, lipid peroxidation and reactive oxygen species (ROS) formation.

b) Catechin (catechin, epicatechin and EGCG) effectively prevent against dicarbonyl (methylglyoxal or glyoxal) induced protein carbonylation in a cell free model system, using bovine serum albumin. These catechins also decrease protein carbonylation and cell death induced by methylglyoxal or glyoxal in isolated hepatocytes, likely by trapping the reactive dicarbonyls, thereby preventing protein carbonyl (Schiff base) formation.

Chapter 3 – Materials and Methods

2.1 Chemicals

Glyoxal, tertiary-butyl hydroperoxide, trichloroacetic acid (TCA), 2,4-dinitrophenyl hydrazine (DNPH), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), (+)-catechin, (-)-epicatechin, epigallocatechin gallate (EGCG), dimethyl sulfoxide (DMSO), methylglyoxal, 1-bromoheptane, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), Trolox, p-coumaric acid, caffeic acid, ellagic acid, quercetin, and all other chemicals were of the highest quality commercially available were purchased from Sigma Chemical Co. (St. Louis, MO). Type II Collagenase (from *Clostridium histoloticum*), bovine serum albumin (BSA) and N-2-hydroxyethylpiperazine-N'-2-2ethanesulfonic acid (HEPES) were purchased from Boehringer Mannheim (Montreal, Canada). Thiobarbituric acid (TBA) was purchased from Fisher Scientific. Whole walnuts and whole hazelnuts were purchased from Loblaws Companies Ltd (Toronto, Canada).

2.2 Preparation of crude walnut and hazelnut extracts

Crude nut extracts were prepared by the following method. Whole walnuts and hazelnuts (kernel with skin) were purchased from Loblaws, Toronto, Ontario, and stored in the dark at 2 °C until extract preparation. The following protocol was used to prepare extracts: 6 grams of nuts (walnuts or hazelnuts) were crushed into a fine powder with a glass mortar and pestle. The ground nut powder was added to 24 mL of either methanol (100 %), ethyl acetate, or Millipore water in 125 mL Erlenmeyer flasks. The flasks were sonicated for 60 minutes at a temperature between 5 °C – 10°C. The mixture was separated by vacuum

filtration, and the liquid extract (filtrate) was collected; for methanolic extracts or extracts made from ethyl acetate, methanol or ethyl acetate were evaporated using a constant stream of nitrogen gas at room temperature. For the water nut extracts, the collected filtrates were frozen at –20 °C, and lyophilized using a VIRTIS Benchtop. Once the solvents were evaporated from the filtrates, the extracts were weighed and frozen at –70 °C until further use. The extracts were used within 2 months, and were dissolved in either Millipore water, methanol or DMSO. Methanol or DMSO alone did not affect hydroperoxide or glyoxal or methylglyoxal cytotoxicity, nor did they elicit protective effects against the toxins used.

2.3 Determination of the Ferric-reducing antioxidant power (FRAP) of hazelnut extracts, walnut extracts and antioxidants

The ferric reducing/antioxidant power (FRAP) assay directly measures antioxidants or reductants in a sample and has been noted as a suitable method for assessing total antioxidants in plants. Therefore, in the present study, the FRAP assay was used to determine the antioxidant capacities of walnut and hazelnut extracts, and of several polyphenolic antioxidants, some of which are commonly found in plants. The FRAP assay is based on the ability of antioxidants to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) at low pH, which has an intense blue colour when in complex with TPTZ (Fe^{2+}/TPTZ), with an absorption maximum at 593 nm (Benzie and Strain 1996). Acetate buffer (25 mL, 300 mM) was prepared (pH 3.6) by combining 3.1 g of sodium acetate ($C_2H_3NaO_2$*3 H_20), 16 mL of acetic acid ($C_2H_4O_2$) and 986 mL of Millipore water. Secondly, 2.5 mL of tripyridyl-s-triazine (TPTZ) solution was prepared using 40 mM

HCl with 10 mM TPTZ. Thirdly, 2.5 mL of 20 mM ferric chloride solution was prepared. All three solutions were combined for a total of 30 mL of FRAP reagent. A calibration curve was determined in 10 wells using a range of concentrations (100-1000 μM) of ferrous sulphate (Fe_2SO_4*7 H_20). The FRAP reagent (300 μL) was incubated at 37 °C in a plate reader for several minutes after which a blank reading was recorded at 593 nm. The nut extract samples (10 μL) and water (30 μL) were added to the FRAP reagent and the absorbance was read at 593 nM at 4, 8, and 30 minutes (Benzie and Strain 1996). FRAP values are expressed in μM concentrations of ferrous sulphate (FeSO4) obtained from a linear calibration curve.

2.4 Microsomal Preparation

Hepatic microsomes were prepared from adult male Sprague-Dawley rats, by differential centrifugation as described by Dallner (Dallner 1978). The microsomal pellet was suspended in sterile potassium phosphate buffer with KCL solution (50 mM KH_2PO_4 and 0.23 % w/v KCl, pH 7.4) before storage at -70°C. Quantification of microsomal protein content was determined by the bicinchoninic acid method (Stoscheck 1990).

2.5 Determination of microsomal lipid peroxidation and antioxidant activity

Microsomal lipid peroxidation was determined by measuring the amount of malondidaldehyde (MDA), a thiobarbituric acid reactive substance (TBARS) at 532 nm, formed during the decomposition of lipid hydroperoxides using a Pharmacia Biotech Ultraspec 1000. A reaction mixture containing 1 ml of microsomes (1 mg/ml protein) in 0.1 M sodium phosphate buffer (pH 7.4), 500 μM tertiary-butyl hydroperoxide and

hazelnut or walnut extracts (50, 100, or 150 μg/ml). Samples were incubated at 37 °C and treated with TCA (250 μL, 70 % w/v) to stop the reaction and lyse the cells at 30 or 90 min, and then 1 ml of TBA (0.8 % w/v) was added to the cells to determine the formation of lipid peroxidation products. The suspensions were incubated in a boiling water bath for 20 minutes, after which they were cooled on ice for 5 minutes, and centrifuged at 4000 RPM for 5 minutes. The supernatant was read at 532 nm using a Pharmacia Biotech Ultrospec 1000 (Smith et al. 1982). The concentrations of TBARS were expressed as μM concentration of malondialdehyde (MDA) which was calculated by using a molar extinction coefficient of 1.56×10^5 $mol^{-1} \cdot cm^{-1}$. None of the nut extracts or compounds used reacted with TBA or absorbed at 532 nm.

2.6 Determination of protein carbonyl content of Bovine Serum Albumin (BSA)

In a cell free assay, the total protein bound carbonyl content of bovine serum albumin (BSA) was determined by derivatizing the protein carbonyl adducts with 2,4-dinitrophenylhydrazine (DNPH) which forms a stable dinitrophenyl (DNP) hydrazone product that is then read spectrophotometrically. BSA (2 mg/ml) was prepared in 50 mM phosphate buffer (pH 7.4). BSA (0.5 ml) was incubated for a 1 hour at room temperature with 0.5 ml of DNPH (0.1 % w/v) in 2 N hydrochloric acid (HCL). 1 ml of trichloroacetic acid (TCA) (20 % w/v) was added to the suspension to stop the reaction. The sample was centrifuged at 500 rpm to obtain a pellet, and the supernatant was removed. DNPH was removed by extracting the pellet three times using 0.5 ml of ethyl acetate:ethanol (1:1 v/v) solution. After the extraction, the pellet was dried under a gentle stream of nitrogen and dissolved in 1 ml of Tris-buffered 8.0 M guanidine-HCL (pH 7.2).

The solubilized hydrazones were measured at 370 nm. The concentration of DNPH derivatized proteins was determined by the molar extinction coefficient of 22, 000 $M^{-1}cm^{-1}$ (Hartley et al. 1997).

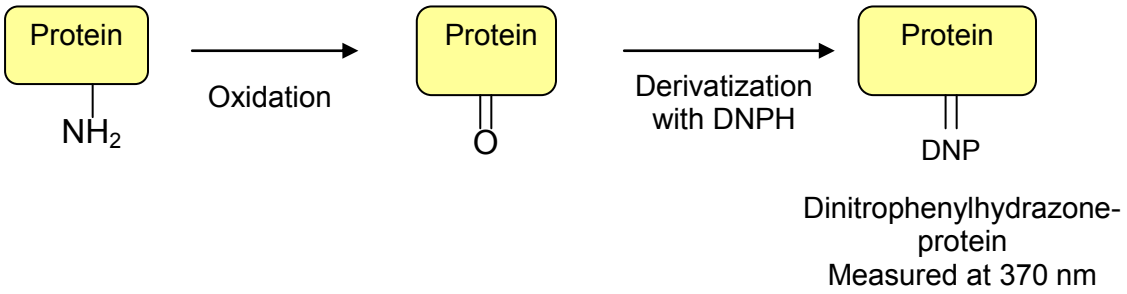


Figure 2-1: Protein Carbonyl Content detected as Adducts of Dinitrophenylhydrazine (DNPH) (DNPH = 1,2-dinitrophenylhydrazine)

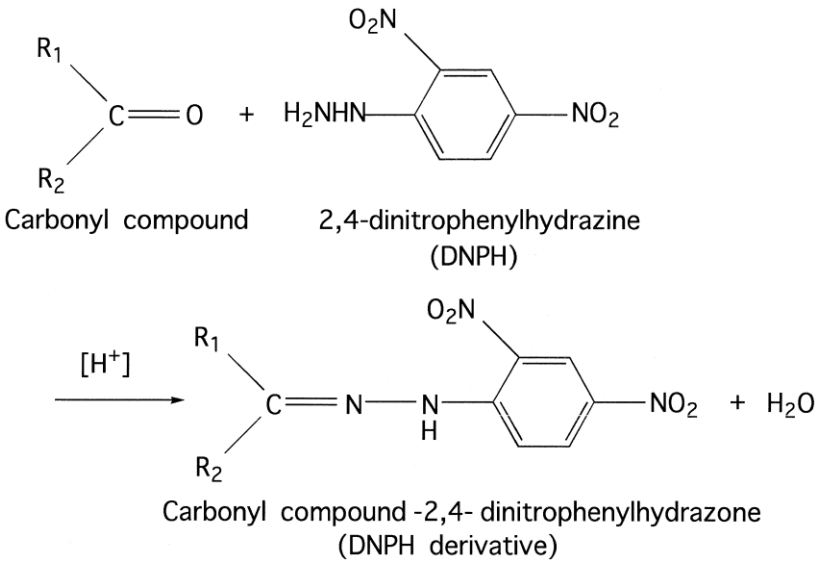


Figure 2-2: Derivatization Reaction occurring between Carbonyl Species and DNPH (Sakuragawa et al. 1999)

2.7 Animal Treatment

Male Sprague-Dawley rats weighing 275-300 grams (Charles River Laboratories) were housed in ventilated plastic cages over PWI 8-16 hardwood bedding, with 12 air changes per hour, 12 hour light photoperiod (lights on at 8:00 am) and an environmental temperature of 21-23 ° C. The animals were fed a standard chow diet and water ad libitum.

2.8 Hepatocyte isolation and preparation

Hepatocytes were isolated by collagenase liver perfusion as described previously by Moldeus et al (Moldeus et al. 1978). Isolated hepatocytes (10^6 cells/ml) (10 ml) were suspended in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continually rotating 50 ml round-bottom flasks, under an atmosphere of 95 % O_2 and 5 % CO_2 in a water bath of 37 ° C for 30 minutes. Stock solutions of chemicals were prepared immediately prior to use, either in Millipore water, DMSO or methanol.

2.9 Hepatocyte Viability

Hepatocyte viability was tested microscopically by plasma membrane disruption, as determined by the trypan blue (0.1 % w/v) exclusion test (Moldeus et al. 1978). Hepatocyte viability was assessed at 30 minutes and then hourly up to 180 minutes, and the cells were at least 80-90 % viable before use. Lethal toxin doses which caused approximately 50 % cell death at 2 hours were used for the toxic chemicals (glyoxal, methylglyoxal or tertiary-butyl hydroperoxide).

2.10 Lipid peroxidation determination in hepatocyte

Lipid peroxidation was assayed by measuring the amount of thiobarbituric acid reactive substances (TBARS), formed during the lipid hydroperoxide decomposition, mostly formed from malondialdehyde (MDA) with the pink adduct being measured at 532 nm. 1 ml aliquots of hepatocyte suspension (10^6 cells/ml) were treated with trichloroacetic acid (TCA) (250 uL, 70 % w/v) to stop the reaction and lyse the cells. After this 1 ml of thiobarbituric acid (TBA) (0.8 % w/v) was added to the cells to determine the formation of lipid peroxidation products. Suspensions were incubated in a boiling water bath for 20 minutes, after which they were cooled on ice for 5 minutes, and centrifuged at 4000 RPM for 5 minutes. The supernatant was read at 532 nm using a Pharmacia Biotech Ultrospec 1000 (Smith et al. 1982). The concentrations of TBARS were expressed as μM concentration of MDA and calculated by using a molar extinction coefficient of 1.56 x 10^5 $mol^{-1} \cdot cm^{-1}$. None of the nut extracts or compounds used reacted with TBA or absorbed at 532 nm.

2.11 Determination of reactive oxygen species

Hepatocyte reactive-oxygen species (ROS) generation induced by tertiary butyl-hydroperoxide was determined by adding dichlorofluorescin diacetate (DCFH-DA) to the hepatocyte incubate. DCFH-DA penetrates hepatocytes and is hydrolyzed to form non-fluorescent dichlorofluorescin (DCFH). DCFH is then oxidised by ROS to form the highly fluorescent dichlorofluorescein (DCF) which effluxes the cell. After incubation with tertiary butyl hydroperoxide, 1 ml samples of hepatocytes were withdrawn at 90 minutes and centrifuged at 50 x g for 1 minute. The cells were resuspended in Krebs-

Henseleit buffer and 1.6 μM DCFH-DA was added (Shangari and O'Brien 2004). Cells were allowed to incubate at 37 ° C for 10 minutes. The fluorescent intensity of DCF was determined by measuring the excitation and emission wavelengths, which were 490 and 520 nm, respectively.

2.12 Determination of protein carbonyl content of hepatocytes

The total protein bound carbonyl content was determined by derivatizing the protein carbonyl adducts with 2,4-dinitrophenylhydrazine (DNPH). Briefly, 0.5 mL of cells (0.5 x 10^6 cells) was incubated for 1 hour at room temperature with 0.5 ml of DNPH (0.1 % w/v) in 2 N HCL. 1 ml of TCA (20 % w/v) was added to the suspension to stop the reaction. The sample was centrifuged at 500 rpm to obtain the cellular pellet, and the supernatant was removed. DNPH was removed by extracting the pellet three times using 0.5 ml of ethyl acetate:ethanol (1:1, v/v) solution. After extractions, the pellet (cellular protein) was dried under a gentle stream of nitrogen and dissolved in 1 ml of Tris-buffered 8.0 M guanidine-HCL (pH 7.2). The solubilized hydrazones were measured at 370 nm. The concentration of DNPH derivatized proteins was determined by the molar extinction coefficient of 22, 000 $M^{-1}cm^{-1}$ (Hartley et al. 1997).

2.13 Preparation of Glutathione (GSH) depleted hepatocytes

GSH-depleted hepatocytes were prepared by incubating 200 μM of 1-bromoheptane for 30 minutes with the hepatocyte incubate, as described by Khan and O'Brien (Khan and O'Brien 1991). Gluthathione-S-transferase transfers the heptyl group of 1-bromoheptane to GSH to form heptyl-S-glutathione and releases bromide (Figure 2-3). Bromoheptane,

unlike other GSH-depleting agents, is not toxic to hepatocytes, even at ten times the concentration which is required to deplete hepatocyte GSH (Khan and O'Brien 1991). Bromoheptane also depleted hepatocyte GSH much more rapidly and completely compared to other GSH-depleting agents (Khan and O'Brien, 1991).

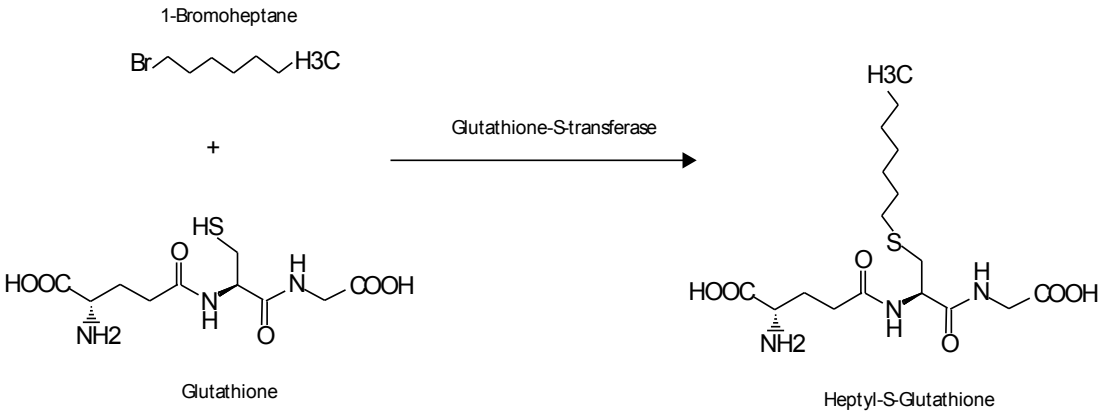


Figure 2-3: 1-Bromoheptane depletes GSH in hepatocytes by a GSH-s-transferase catalyzed heptyl-s-gluthathione formation. Gluthathione-S-transferase transfers the heptyl group of 1-bromoheptane to GSH to form heptyl-S-glutathione and releases bromide.

2.14 Ultraviolet Spectrophotometric Evidence of a Catechin Dicarbonyl Adduct

Ultraviolet (UV) spectrophotometric scans were conducted using a Pharmacia Biotech Ultrospec 1000, in order to detect any possible changes in the spectrum for catechin, upon addition of methylglyoxal or glyoxal. Catechin (300 μM), dissolved in Millipore water, was added to 50 mM sodium phosphate buffer (pH 7.4) and was scanned over a range of 200 – 700 nm, every 6 minutes for a 60 minute period. After catechin alone was scanned, either methylglyoxal (15 mM) or glyoxal (15 mM) was added to the cuvette, and subsequently scanned periodically (every 6 minutes) over a 60 minute period.

2.15 Statistical Analyses

Statistical analyses were performed by conducting a one-way ANOVA test and its significance was assessed by employing Tukey's *post hoc* test. Results are presented as the mean ± standard error (SE) from three separate experiments, and a probability of less than 0.05 was considered significant. SPSS version 16.0 for Windows was used as the statistical software for analyses.

Chapter 4 – Results

3.1 Percent yields of walnut and hazelnut extracts

The percent yields of solids of the three different walnut and three different hazelnut extracts is shown in Table 3-1. Among the walnut extracts, the walnut ethyl acetate extract showed the highest percent yield (63 %), followed by the walnut methanolic extract (42 %) and the walnut water extract (33 %). Among the hazelnut extracts, the hazelnut ethyl acetate extract showed the highest percent yield (74 %), followed by the hazelnut methanolic extract (24 %) and the hazelnut water extract (10 %). The ethyl acetate extracts obtained for each nut yielded yellow oils. Among the walnut extracts, the percent yields were significantly different between the walnut ethyl acetate

Table 3-1: Percent yields * of hazelnut and walnut extracts

Prepared extracts	Percent Yield
hazelnut ethyl acetate extract	74 ± 10.5 †, ††
hazelnut methanolic extract	10 ± 4.2 ‡
hazelnut water extract	24 ± 5.5
walnut ethyl acetate extract	63 ± 7.6 ^
walnut methanolic extract	42 ± 7.0
walnut water extract	33 ± 5.3

* Percent yields were determined by measuring the amount of solid recovered after the extraction process compared to the initial weight of nuts measured. 6 grams of each nut was used for the extraction process, which is described in the Materials and Methods section

† significant compared to hazelnut water extract

†† significant compared to hazelnut methanolic extract

^ significant compared to walnut water extract

‡ significant compared to the same extract of a different nut

extract and the a) walnut water extract ($p=0.02$) and b) the walnut methanolic extract ($p=0.026$) whereas, the hazelnut ethyl acetate extract percent yield was significantly different compared to the hazelnut methanolic extract yield ($p<0.001$) and the hazelnut water extract ($p<0.001$). When comparing the same extract of the different nuts (i.e. hazelnut water vs. walnut water extract), the methanolic extracts between the two nuts had significantly different percent yields ($p=0.001$), whereas the percent yields of the water extracts or the ethyl acetate extracts of the nuts were not significantly different ($p=0.661$; $p=0.445$, respectively).

3.2 FRAP (ferric reducing/antioxidant power) of Walnut and Hazelnut extracts and some polyphenolic compounds

The FRAP assay is a commonly used method for determining the antioxidant or reducing potential of a sample, and has been used often in determining antioxidant capacity of plants or plant extracts. As shown in Figure 3-1, the ferric reducing/antioxidant power (FRAP) of hazelnut and walnut extracts and various antioxidants was determined. The overall order of reducing ability of the nut extracts was the following, from the highest reducing capacity to the lowest reducing capacity: walnut methanolic extract > walnut water extract > hazelnut methanolic extract > walnut ethyl acetate extract > hazelnut water extract > hazelnut ethyl acetate extract after 8 minutes of starting the reaction. At the 4 minute reading, a similar trend was observed, except for the hazelnut ethyl acetate extract which had a negative FRAP value, indicating an initial lack of reducing ability or antioxidant power. After a 30 minutes, a similar trend was observed compared to the 8 minute readings, except for the reducing power of the hazelnut water extract which was greater than the walnut ethyl acetate extract.

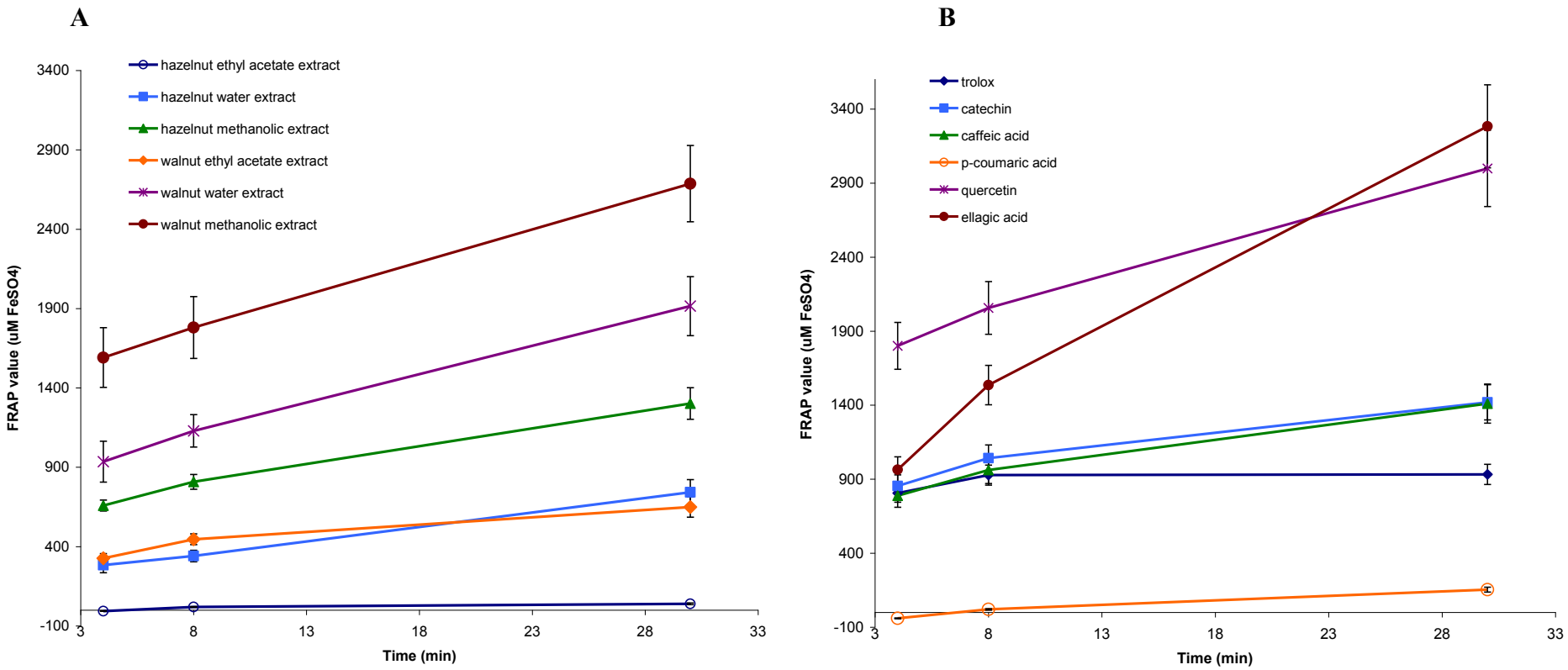


Figure 3-1: FRAP values for Hazelnut and Walnut extracts (A); FRAP values for Antioxidants (B)

The FRAP assay is based on the reduction of ferric iron (Fe^{3+}) to the ferrous form (Fe^{2+}), which complexes with TPTZ to form a blue Fe^{2+}-TPTZ complex. The FRAP value is determined by measuring the increase in absorbance at 593 nm. FRAP values are expressed in µM concentrations of FeSO4 obtained from a linear calibration curve. Means ± SE for three separate experiments are given.

* Nut extract concentrations are 200 µg/ml

** Antioxidant concentrations are 10 µM

The differences in reducing power observed over time indicate that the reducing/antioxidant ability of the nut extracts was dependent on time. The time dependence effect for the FRAP value has been previously noted for some antioxidants, as they may require more time to reduce ferric iron to the ferrous form, particularly for carotenoids or thiols (Magalhaes et al. 2008). The increases in FRAP values over time for all walnut and hazelnut extracts ranged from approximately two to three fold. Catechin, caffeic acid, and quercetin elicited nearly two fold increases in FRAP values while ellagic acid showed a 3.4 fold increase in the FRAP value between the 4 and 30 minute readings. Trolox (a water soluble derivative of vitamin E) and *p*-coumaric acid only slightly increased the reducing abilities over time. After 30 minutes, the order of reducing ability from highest reducing to lowest reducing capacity for the antioxidants was: ellagic acid > quercetin > catechin > caffeic acid > trolox > *p*-coumaric acid. Quercetin initially showed a relatively fast reaction as it had a much higher reducing capacity than ellagic acid after 4 minutes (~ by two fold), while ellagic acid showed a slower reducing reaction. When comparing the antioxidant power of all nut extracts and the polyphenolic antioxidants (after 30 minutes), the overall order of reducing ability was the following, from highest antioxidant power to lowest antioxidant power: ellagic acid > quercetin > walnut methanolic extract > walnut water extract > catechin > caffeic acid > hazelnut methanolic extract > trolox > hazelnut water extract > walnut ethyl acetate extract > *p*-coumaric acid > hazelnut ethyl acetate extract.

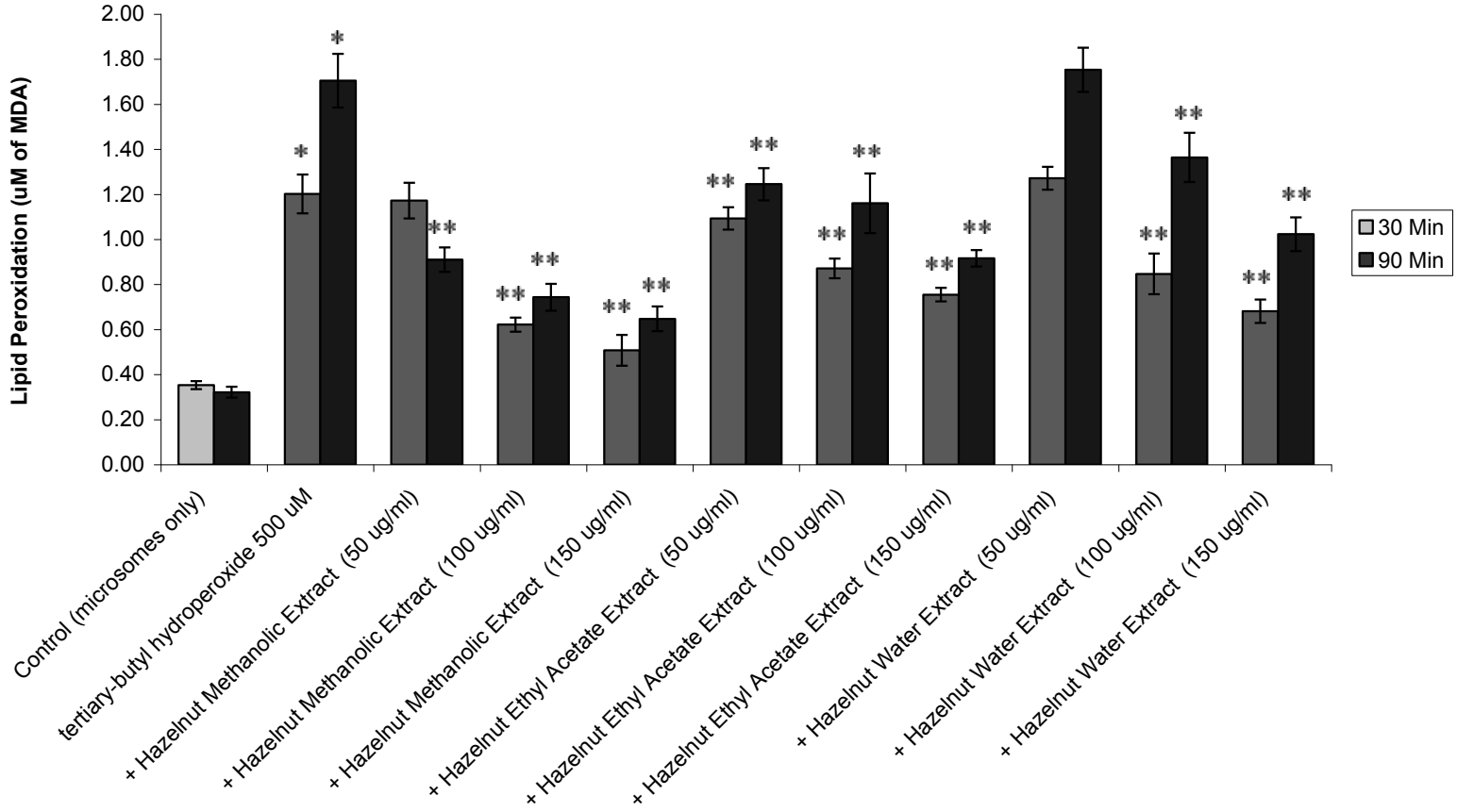
3.3 Dose response protection by three different hazelnut extracts against lipid peroxidation in rat liver microsomes

Tertiary-butyl hydroperoxide (TbuOOH) was used as an oxidizing agent to induce oxidative stress induced lipid peroxidation in rat liver microsomes. Figure 3-2 shows a dose response effect of three different hazelnut extracts, on the protection of lipid peroxidation in rat liver microsomes. The protective effect of hazelnut extracts against TbuOOH-induced lipid peroxidation was, in order of the most protective to least protective: methanolic extract > ethyl acetate extract > water extract, and each extract elicited protection in a dose response manner. At the lowest concentration tested (50 μg/ml), the hazelnut methanolic extract was more effective than the hazelnut ethyl acetate extract against lipid peroxidation after 90 minutes compared to the hydroperoxide (TbuOOH, 500 μM) by 47 % and 27 % respectively, while the water extract at 50 μg/ml failed to protect at 30 or 90 minutes but protected by 20 % at a concentration of 100 μg/ml after 90 minutes. The dose response protection against lipid peroxidation by hazelnut extracts indicates that these extracts act as antioxidants. All extracts significantly reduced lipid peroxidation, except for the lowest concentration (50 μg/ml) of the hazelnut water extract, which did not elicit protection.

3.4 Dose response protection by walnut extracts against lipid peroxidation in rat liver microsomes

The ethyl acetate, methanolic and water extracts of walnuts were also investigated for their ability to elicit protection against TbuOOH-induced lipid peroxidation in rat liver microsomes, as determined by the TBARS assay (Figure 3-3). The order of

Figure 3-2: Hazelnut Extracts Protect against Lipid Peroxidation in Rat Liver Microsomes



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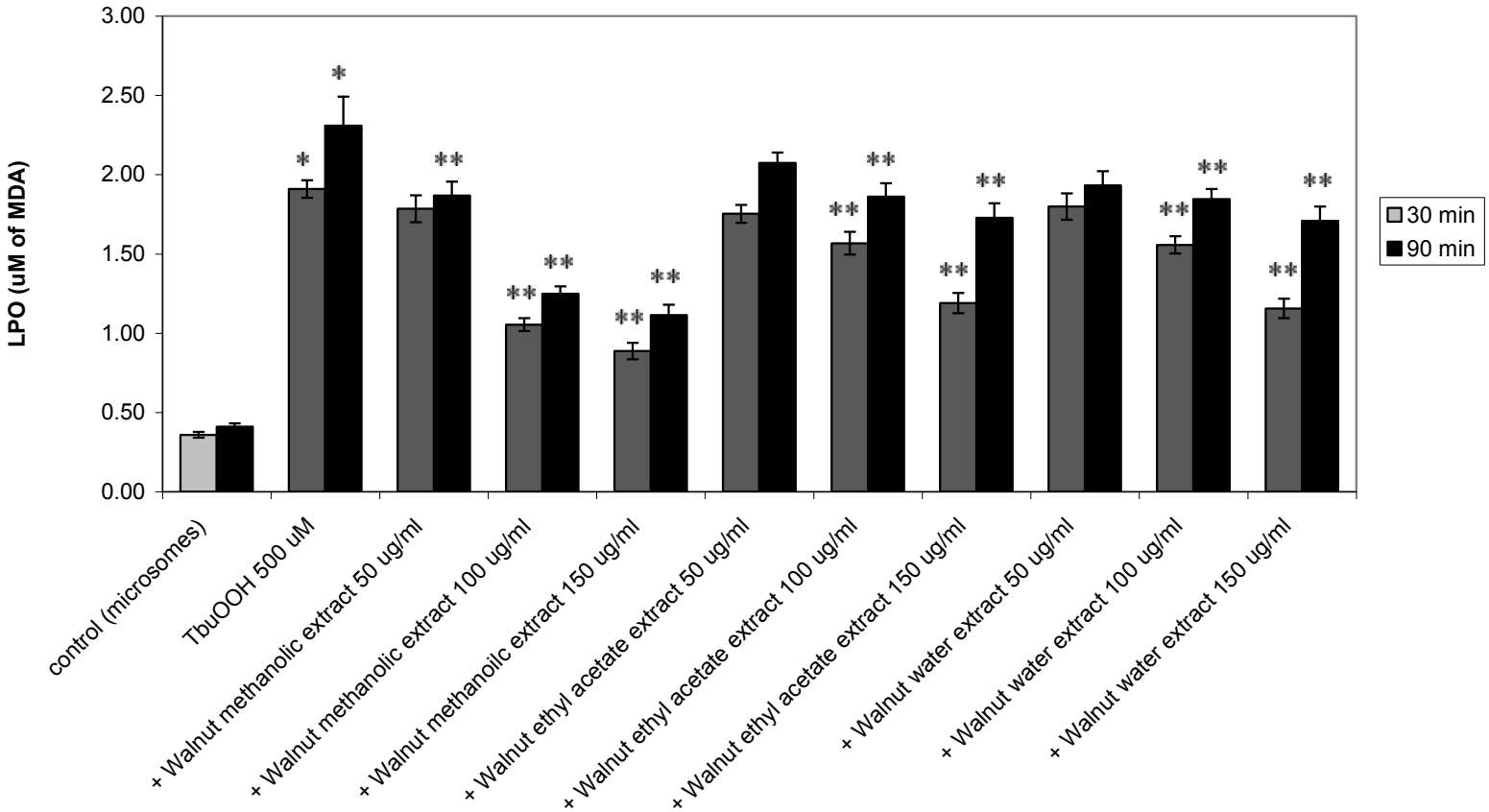
Rat liver microsomes (1mg/mL) in 0.1 M sodium phosphate buffer (pH 7.4) were incubated at 37 ° C and the reaction was stopped at the specific time points. Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances as μM concentration of malondialdehyde (ε = 1.56 x 105 M^{-1} cm^{-1}).

[a] Significant as compared to control

[b] Significant as compared to tertiary-butyl hydroperoxide 500 μM

Figure 3-3: Walnut Extracts protect against Lipid Peroxidation in Rat Liver Microsomes



Rat liver microsomes (1mg/mL) in 0.1 M sodium phosphate buffer (pH 7.4) were incubated at 37 ° C and the reaction was stopped at the specific time points. Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances as µM concentration of malondialdehyde (ε = 1.56 x 105 M^{-1} cm^{-1}).

[a)] Significant as compared to control

[b)] Significant as compared to tertiary-butyl hydroperoxide 500 µM

protection against lipid peroxide formation in microsomes was the following, from most protective to least protective, after 90 minutes: walnut methanolic extract > walnut water extract > water ethyl acetate extract. All three walnut extracts elicited a dose-response protection, where the highest protective effect was observed in the highest concentration used (150 μg/ml) for each extract, and the methanolic extract elicited protection against lipid peroxidation by 51 %, whereas, 26 % and 25 % decreases were observed for water and ethyl acetate extracts after 90 minutes, respectively. At the lowest concentration tested, (50 μg/ml), the protection ranged from approximately 10 – 19 % after 90 minutes, and the ethyl acetate showed the lowest protection (10 %) against lipid peroxidation, whereas the methanolic extract elicited the best protection (18 %) and the water extract protected by 16 %. Most concentrations for the three extracts (50 μg/ml, 100 μg/ml and 150 μg/ml) showed significant reductions in lipid peroxidation after 90 minutes, except for the water extract and the ethyl acetate extracts at the lowest concentration tested (50 μg/ml).

3.5 Walnut and Hazelnut Extracts: *In vitro* Protection against tertiary butyl hydroperoxide induced cytotoxicity, lipid peroxidation and ROS formation in primary rat hepatocytes (Oxidative Stress Model)

Three hazelnut extracts, as well as three walnut extracts, (extract concentrations of 200 μg/ml) were tested and compared for cytoprotection against oxidative stress cell death, lipid peroxidation and ROS formation in primary hepatocytes (Table 3-2). The order of protection against cell death was, from most protective to least protective after 120 minutes: walnut methanolic extract > walnut water extract > walnut ethyl acetate extract (oil) > hazelnut water extract > hazelnut methanolic extract. The non-polar ethyl

Table 3-2: Nut Extracts Prevent Cell Death in Rat Hepatocytes

Compounds added	Percent Cytotoxicity (% trypan blue uptake)			Lipid Peroxidation (nmol/10^6 cells)†	ROS formation (FI units)
	60 min	120 min	180 min	90 min	90 min
Control	16 ± 1[b)]	18 ± 2[b)]	26 ± 3[b)]	0.31 ± 0.01[b)]	98 ± 3.94[b)]
+ Tertiary butyl hydroperoxide 500 µM	35 ± 3[a)]	80 ± 3[a)]	83 ± 4[b)]	2.38 ± 0.12[a)]	252 ± 5.03[a)]
+ Walnut ethyl acetate extract (200 µg/ml)	31 ± 6[a)]	29 ± 3[a) b)]	47 ± 2[a) b)]	0.97 ± 0.07[a) b)]	157 ± 6.28[a) b)]
+ Walnut methanolic extract (200 µg/ml)	20 ± 5[b)]	20 ± 1[b)]	21 ± 1[b)]	0.45 ± 0.04[b)]	73 ± 2.34[a) b)]
+ Walnut water extract (200 µg/ml)	27 ± 1	24 ± 4[b)]	38 ± 1[a) b)]	0.55 ± 0.02[a) b)]	108 ± 3.12[a) b)]
+ Hazelnut ethyl acetate extract (200 µg/ml)	40 ± 3[a)]	78 ± 2[a)]	93 ± 4[a) b)]	1.92 ± 0.13[a) b)]	225 ± 9.00[a) b)]
+ Hazelnut methanolic extract (200 µg/ml)	28 ± 2[a)]	34 ± 8[a) b)]	61 ± 2[a) b)]	1.00 ± 0.03[a) b)]	147 ± 5.86[a) b)]
+ Hazelnut water extract (200 µg/ml)	30 ± 8[a)]	31 ± 2[a) b)]	50 ± 3[a) b)]	1.09 ± 0.06[a) b)]	176 ± 5.16[b)]

Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37 °C in rotating round bottom flasks with 95 % O_2 and 5 % CO_2 in Krebs-Henseleit buffer (pH 7.4). † Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances as nmol/10^6 cells of malondialdehyde ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$). Means ± SE for three separate experiments are given. All extracts are 200 µg/ml.

a) Significant as compared to control ($p < 0.05$)

b) Significant as compared to tertiary butyl hydroperoxide 500 µM ($p < 0.05$)

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acetate hazelnut extract (oil) failed to prevent cell death. The same order of protection occurred later on in the experiment, after 180 minutes, however the hazelnut ethyl acetate extract resulted in a slight increase in cell death. The order of protection by nut extracts against oxidative stress induced lipid peroxidation in hepatocytes was the same order of protection elicited against cell death, except for the hazelnut methanolic extract which elicited better protection than the hazelnut water extract. The hazelnut ethyl acetate extract was least protective against lipid peroxidation (19 %) compared to the other extracts (protection ranged from approximately 54 to 81 %); however, all extracts were significantly protective. When tested against ROS formation in hepatocytes, all six extracts significantly prevented ROS formation, in the order of most protective to least protective: walnut methanolic extract (71 %) > walnut water extract (57 %) > hazelnut methanolic extract (42 %) > walnut ethyl acetate extract (38 %) > hazelnut water extract (30 %) > hazelnut ethyl acetate extract (11 %).

Summary: In all three experiments with primary hepatocytes, it was evident that the methanolic and water extracts had superior protective effects overall when compared to the ethyl acetate extracts of each nut, with the hazelnut ethyl acetate extract having the minimal or no protection.

3.6 The Effect of Catechins on Protein Carbonylation induced by MGO or GO in a Cell Free System (Bovine Serum Albumin)

The effectiveness of pure catechin, epicatechin and EGCG against protein carbonylation was compared in a cell free model using bovine serum albumin (BSA),

under physiological temperature (37 °C) and pH (pH 7.4). Protein carbonylation was induced by either glyoxal (GO) or methylglyoxal (MGO) in this system.

In the first experiment, each of the three catechins was tested against an equimolar concentration of either glyoxal or methylglyoxal (Figure 3-4), where catechins were added to the BSA incubate at the same time as either dicarbonyl (GO or MGO). None of the three catechins significantly reduced GO induced protein carbonylation over time (Figure 3-4 A), although each compound did prevent protein carbonylation to a minimal extent, and the order from most protective to least protective was: catechin, epicatechin > EGCG. All three catechins, however, significantly reduced protein carbonylation induced by MGO after 2 hours (Figure 3-4 B). In particular, catechin was highly and significantly effective at reducing carbonylation at 0 minutes, and its protection significantly differed from the small reduction elicited by epicatechin ($p=0.049$). The order of protection against MGO, from most protective to least protective was: catechin > epicatechin > EGCG. The decrease elicited by epicatechin or catechin against MGO, was significantly lower, compared to the decrease elicited by EGCG ($p=0.001$). It is interesting to note that, although both epicatechin and catechin elicited similar reductions in protein carbonylation against MGO after 2 hours, catechin was initially much more effective, although not significant, whereas epicatechin showed a slower rate of protection. Also, the amounts of protein carbonylation induced by GO or MGO were similar.

In the second experiment using BSA, GO or MGO were incubated with BSA for a 120 minute period, after which, either one of the three catechins was added to the BSA-dicarbonyl mixture at 0 minutes (Figure 3-5). The compounds were added after the

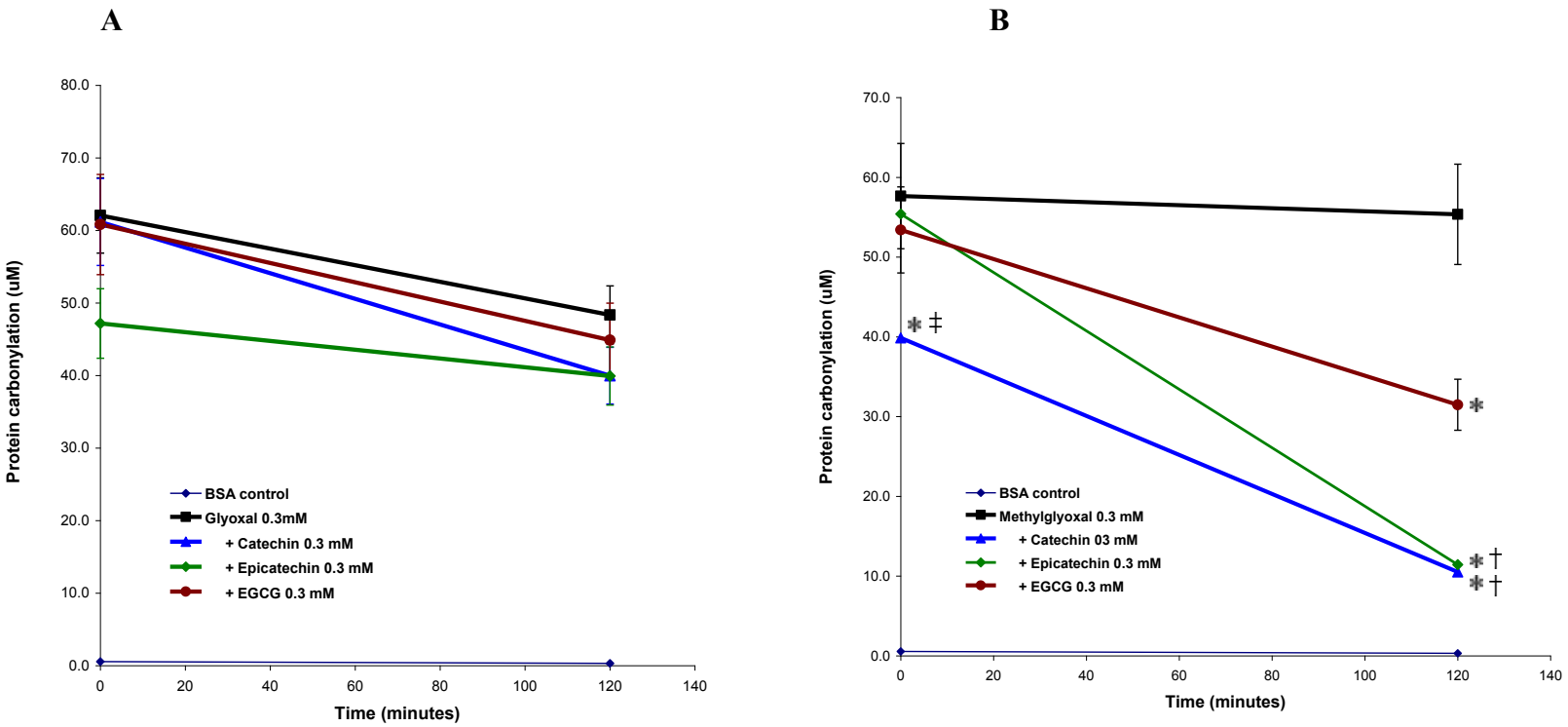

Figure 3-4: Catechins prevented Protein Carbonylation induced by Glyoxal (A) or Methylglyoxal (B) in Serum Albumin

Glyoxal or methylglyoxal with or without catechin, epicatechin or EGCG were incubated with BSA (2 mg/ml) in 50 mM phosphate buffer (pH 7.4) at 37 °C. Protein carbonylation was determined by measuring DNPH-derivatized samples (μM), measured at an absorbance of 370 nm. The Molar Extinction Coefficient of 22,000 M^{-1} cm^{-1} was used to calculate the carbonyl content. Means ± SE for three separate experiments are given.

^ Compounds were added to the incubate at the same time as glyoxal (A) or methylglyoxal (B).

* Significant compared to dicarbonyl, glyoxal (A) or methylglyoxal (B)

† Significant compared to EGCG (Graph B)

‡ Significant compared to EGCG or Epicatechin (Graph B)

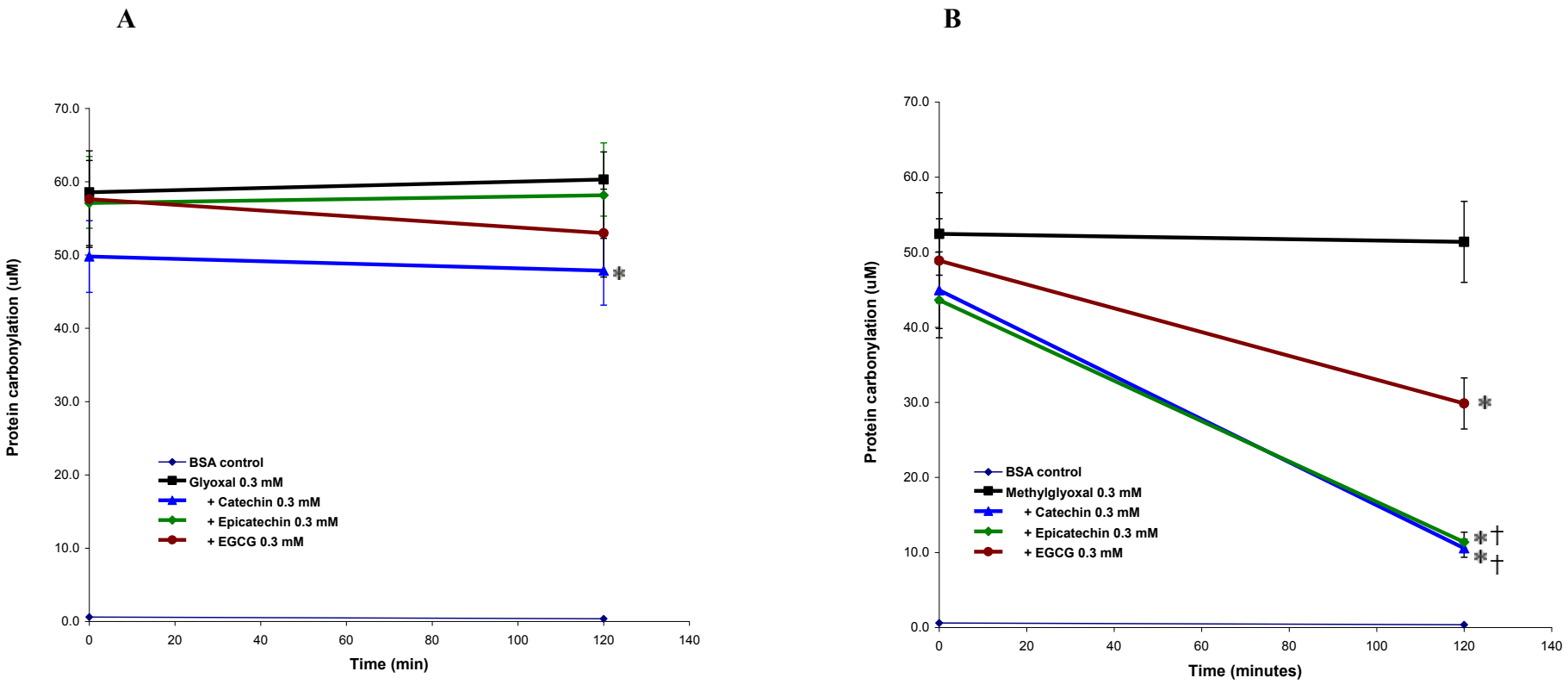

Figure 3-5: Catechins reversed Protein Carbonyl (Schiff base) formation induced by Glyoxal (A) or Methylglyoxal (B) in Serum Albumin
Glyoxal (A) or methylglyoxal (B) were incubated with BSA (2 mg/ml) in 50 mM phosphate buffer (pH 7.4) at 37 °C for 120 minutes, after which catechin, epicatechin or EGCG were added to the incubate at 0 minutes. Protein carbonylation was determined by measuring DNPH-derivatized samples (μM), measured at an absorbance of 370 nm. The Molar Extinction Coefficient of 22,000 M^{-1} cm^{-1} was used to calculate the carbonyl content. Means ± SE for three separate experiments are given. ^ Compounds were added to the incubate 2 hours after glyoxal (A) or methylglyoxal (B).
* Significant compared to dicarbonyl, glyoxal (A) or methylglyoxal (B)
† Significant compared to EGCG (Graph B)

dicarbonyls in order to determine if these compounds were effective at reversing early stage protein carbonylation (Schiff base formation), thus a 120 minute period was used to allow GO or MGO to react with BSA. Initially, at 0 minutes, catechin reduced protein carbonylation induced by GO much more effectively, compared to epicatechin or EGCG (Figure 3-5 A). After 120 minutes, the order of protection against GO induced carbonylation was, from most to least protective: catechin > EGCG > epicatechin, with catechin being the only compound to significantly reduce carbonylation ($p=0.042$). Against MGO induced protein carbonylation, all three catechins significantly protected after 120 minutes, where the order of protection was, from most to least protective: catechin, epicatechin > EGCG (Figure 3-5 B). Reductions by both catechin and epicatechin significantly differed compared to the reduction by EGCG ($p=0.001$ for both compounds compared to EGCG). Interestingly, catechin and epicatechin elicited a nearly identical line, and hence protection ability, against MGO induced Schiff Base formation, as shown in Figure 3-5 B. Hence, all three catechin compounds effectively reversed early stage carbonylation induced by MGO, whereas, only catechin effectively reversed protein carbonyl (Schiff base) formation induced by GO. Both dicarbonyls maintained a similar level of protein carbonylation over time, and furthermore, did not significantly differ between each other in the amount of carbonylation (0 minutes, $p=0.856$; 120 minutes, $p=0.259$).

In the third experiment, catechin was further explored in the same cell free model (using BSA), as this compound seemed to elicit the best protection (Figures 3-4 and 3-5), compared to epicatechin and EGCG, as previously mentioned. Catechin was premixed with either glyoxal (GO) or methyglyoxal (MGO) for a 30 minute period, after which, the

compounds were added to BSA. As shown in Figure 3-6, premixing catechin with either GO or MGO significantly reduced protein carbonylation at all time points. Most interesting were the results at 0 minutes, where catechin immediately reduced carbonylation by approximately 43 % and 54 % against GO or MGO induced carbonylation, respectively. In the previous experiments, reductions by catechin were not as great, except for its reduction against MGO (~ 31 %), when added at the same time as MGO, which was a comparable reduction, yet not as effective as premixing catechin with

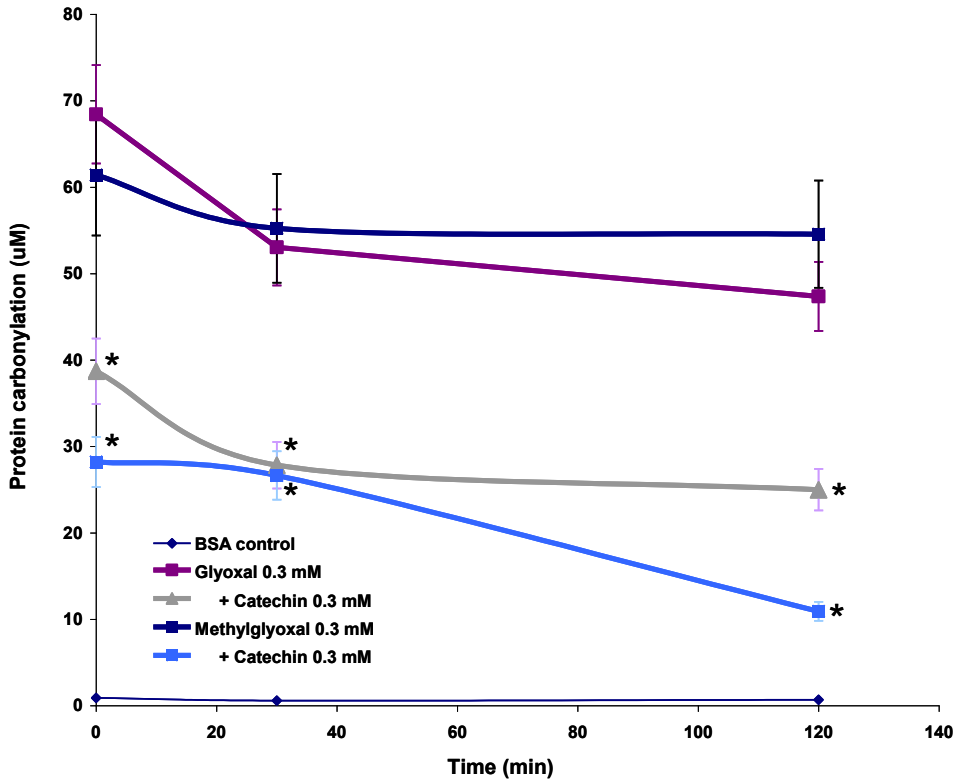

Figure 3-6: Premixing Catechin with Glyoxal or Methylglyoxal decreased Protein Carbonylation in Serum Albumin

Glyoxal or methylglyoxal were premixed for 30 minutes with catechin prior to adding to BSA (2 mg/ml) in 50 mM phosphate buffer (pH 7.4) at 37 °C. Protein carbonylation was determined by measuring DNPH-derivatized samples (µM), measured at an absorbance of 370 nm. The Molar Extinction Coefficient of 22,000 M^{-1} cm^{-1} was used to calculate the carbonyl content. Means ± SE for three separate experiments are given.
* Significant compared to dicarbonyl

MGO. The premix of catechin with either of the dicarbonyls, continued to decrease protein carbonyl formation after 2 hours, suggesting that catechin required more time in order to decrease carbonylation. The results from this experiment, suggest that premixing catechin with dicarbonyls allowed catechin to trap GO and MGO and hence prevented these highly reactive dicarbonyls from carbonylating proteins.

3.7 Effect of Catechin against Glyoxal induced cytotoxicity and protein carbonylation in primary rat hepatocytes

Catechin prevented glyoxal induced cell death, lipid peroxidation, and protein carbonylation, and even elicited protection when added to hepatocytes 30 minutes after glyoxal was added (Table 3-3). When catechin was added either 5 minutes or 30 minutes after glyoxal, only the addition of catechin at 5 minutes after glyoxal significantly protected against cell death (25 % protection), while catechin addition 30 minutes later elicited minimal protection (9 % protection), which was not statistically significant at the latest time point (180 minutes). At 180 minutes, the catechin + glyoxal premix elicited the best cytoprotection against cell death (41 %), compared to the addition of catechin at the same time (34 %) or 5 or 30 minutes after glyoxal addition to hepatocytes (25 %, and 9 % respectively). Only the addition of catechin after 30 minutes of glyoxal addition was significantly different compared to the other times of when catechin was added, in terms of cell death. Catechin significantly reduced lipid peroxidation induced by glyoxal, regardless of when catechin was added, as the protection was not significantly different between the different times of when catechin was added to hepatocytes. The amount of protection against lipid peroxidation ranged from approximately 69 to 77 %.

Catechin also significantly prevented protein carbonyl formation at both 30 and 60 minutes. Additionally, catechin was able to reverse carbonyl formation, as it protected against glyoxal induced carbonylation when added 5 or 30 minutes after glyoxal incubation with hepatocytes, compared to catechin being added at the same time or premixed with glyoxal, which also significantly prevented carbonyl formation. The order of protection against protein carbonylation differed between the 30 and 60 minute time points. Initially the premix of catechin + glyoxal elicited the best protection, followed by the addition of catechin 5 minutes after glyoxal, the addition of the compounds (catechin and glyoxal) to cells at the same time, and finally the least protective was the addition of catechin 30 minutes after glyoxal. However, after 60 minutes, even though the order of protection differed, the least protective result occurred when catechin was added to hepatocytes 30 minutes after glyoxal which was also significantly different from the other times of catechin addition ($p<0.01$), while the order of protection was similar among the different times which catechin was added (premix vs. same time addition $p=0.553$, or premix vs. addition of catechin after 5 min, $p>0.99$).

3.8 Effect of Catechin, Epicatechin or EGCG against glyoxal induced cytotoxicity and protein carbonylation in primary rat hepatocytes

When catechin, epicatechin and EGCG were compared for cytoprotection against glyoxal induced carbonyl stress in hepatocytes, both epicatechin and EGCG were somewhat more protective than catechin against cell death and protein carbonylation although EGCG was more protective at a lower concentration (1 mM) compared to catechin or epicatechin (both at 2.5 mM) (Table 3-4).

Table 3-3: Catechin Prevents Glyoxal induced Cytotoxicity, Lipid Peroxidation and Protein Carbonylation in Primary Rat Hepatocytes

Compounds Added	Percent Cytotoxicity (% trypan blue uptake)			Lipid Peroxidation (nmol/10^6 cells)[†]	Protein Carbonylation (nmol/10^6 cells)[††]	
	60 min	120 min	180 min	90 min	30 min	60 min
control – hepatocytes	21 ± 3	22 ± 3	25 ± 2	0.22 ± 0.01	6 ± 0.2	5 ± 0.2
catechin 10 mM	22 ± 2	23 ± 2[e)]	25 ± 3	0.09 ± 0.01	8 ± 0.2	8 ± 0.2
glyoxal 5 mM	32 ± 3	57 ± 4[d)]	100 ± 2[d)]	2.55 ± 0.11[d)]	410 ± 7.4[d)]	554 ± 15.6[d)]
+ catechin 10 mM	32 ± 5	47 ± 2[d)]	66 ± 4[e)]	0.70 ± 0.02[e)]	319 ± 9[e)]	244 ± 6.9[e)]
+ catechin 10 mM[a)]	30 ± 7	44 ± 5[d)]	75 ± 8[e)]	0.78 ± 0.03[e)]	263 ± 5.2[e)]	258 ± 8.4[e)]
+ catechin 10 mM[b)]	38 ± 2[d)]	66 ± 6[e)]	91 ± 3	0.62 ± 0.02[e)]	339 ± 10.4[e)]	295 ± 9.0[e)]
+ catechin 10 mM[c)]	31 ± 8[d)]	39 ± 5[d)]	59 ± 7[e)]	0.60 ± 0.02[e)]	257 ± 7.9[e)]	251 ± 8.2[e)]

Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37 °C in rotating round bottom flasks with 95 % O_2 and 5 % CO_2 in Krebs-Henseleit buffer (pH 7.4). [†] Lipid Peroxidation was measured as thiobarbituric acid reactive substances as nmol/10^6 cells concentration of malondialdehyde ($\varepsilon = 1.56 \times 10^5$ M^{-1} cm^{-1}). †† Protein Carbonylation was measured as DNPH-derivatized samples as nmol/10^6 cells concentration ($\varepsilon = 2.20 \times 10^4$ M^{-1} cm^{-1}). Means ± SE for three separate experiments are given.

a) compound added to hepatocytes 5 minutes after glyoxal

b) compound added to hepatocytes 30 minutes after glyoxal

c) catechin and glyoxal premixed for 30 minutes prior to addition to hepatocytes

d) Significant as compared to control ($p < 0.05$)

e) Significant as compared to glyoxal 5 mM ($p < 0.05$)

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Furthermore, all three compounds elicited better protection against both outcome measures, cell death and protein carbonylation, when premixed for 30 minutes with glyoxal prior to addition to hepatocytes, compared to not premixing the compounds. However, only epicatechin had significantly better protection against cell death when premixed with GO compared to not premixing with GO ($p<0.01$) at both 120 and 180 minutes. Catechin, either premixed or not-premixed with glyoxal, significantly reduced cell death at 180 minutes, however the effects between the premix and non-premix were not significantly different ($p=0.098$ at 2 hours and $p=0.707$ at 3 hours). EGCG, when premixed or not premixed with glyoxal, was also able to significantly prevent cell death ($p<0.01$ for premix and non-premix) and carbonyl formation ($p<0.01$ for premix and non-premix) compared to the toxic control at 180 minutes for cytotoxicity and 120 min for carbonylation. EGCG elicited better protection against cell death when premixed with glyoxal, although this was not significantly different ($p=0.389$).

All three catechins significantly prevented protein carbonylation at 120 minutes, either when premixed or not premixed with glyoxal. However, catechin and EGCG had significant differences between the premix with GO or not-premixed with GO, suggesting that the premix with GO was able to more effectively reduce protein carbonylation in hepatocytes ($p<0.01$ and $p=0.037$, respectively).

3.9 Effect of Catechin, Epicatechin or EGCG against methylglyoxal induced cytotoxicity and protein carbonylation in GSH depleted primary rat hepatocytes

Catechin, epicatechin and EGCG were tested for their ability to prevent methylglyoxal induced cell death in GSH depleted hepatocytes, as methylglyoxal on its own is not toxic

Table 3-4: Cytoprotection by Catechin, Epicatechin and EGCG by Glyoxal induced Cytotoxicity and Protein Carbonylation

Compounds added	Percent Cytotoxicity (% Trypan blue uptake)			Protein Carbonylation (nmol/10^6 cells)[†]	
	60 min	**120 min**	**180 min**	**45 min**	**120 min**
Control	20 ± 2	23 ± 1	26 ± 2	9 ± 0.4	6 ± 0.3
+ glyoxal 5 mM	22 ± 3	54 ± 3[b)]	100[b)]	544 ± 27.2[b)]	496 ± 24.8[b)]
+ catechin 2.5 mM	24 ± 2	54 ± 4[b)]	81 ± 4[c)]	437 ± 21.8[c)]	321 ± 16.0[c)]
+ catechin 2.5 mM[a)]	24 ± 6	44 ± 4	75 ± 4[c)]	462 ± 23.1[c)]	267 ± 13.4[c)]
+ epicatechin 2.5 mM	26 ± 6	45 ± 5	84 ± 8[c)]	407 ± 20.4[c)]	263 ± 13.2[c)]
+ epicatechin 2.5 mM[a)]	24 ± 6	23 ± 3[c)]	58 ± 5[c)]	363 ± 18.2[c)]	250 ± 12.5[c)]
+ EGCG 1 mM	22 ± 3	47 ± 3[b)]	66 ± 4[c)]	591 ± 29.6[b)]	309 ± 15.4[c)]
+ EGCG 1 mM[a)]	27 ± 3	40 ± 5[b)]	58 ± 4[c)]	447 ± 22.4[c)]	262 ± 13.1[c)]

Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37 °C in rotating round bottom flasks with 95 % O2 and 5 % CO2 in Krebs-Henseleit buffer (pH 7.4). [†] Protein Carbonylation was measured as DNPH-derivatized samples as nmol/10^6 cells ($\varepsilon = 2.20 \times 10^4\ M^{-1}\ cm^{-1}$). Means ± SE for three separate experiments are given.

a) Compound and glyoxal were premixed for 30 minutes prior to addition to hepatocytes

b) Significant as compared to control ($p < 0.05$)

c) Significant as compared to glyoxal 5 mM ($p < 0.05$)

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to hepatocytes because it is rapidly metabolized primarily by glyoxalase I (GLO I), a GSH dependent system (Shangari et al. 2006). GSH was depleted by using a non-toxic concentration (200 μM) of 1-bromoheptane, where cytotoxicity by this bromoalkane has been previously observed to occur at concentrations above 3 mM (Khan & O'Brien 1991). Methylglyoxal becomes toxic to hepatocytes when GSH is depleted. The three catechins were also compared for whether premixing each catechin with methylglyoxal was more effective than adding both compounds to the hepatocyte incubate at the same time (Table 3-5). Catechin, epicatechin and EGCG significantly reduced cell death, when premixed with methylglyoxal for 30 minutes or when added to hepatocytes at the same time as methylglyoxal, except for epicatechin, which, when added to hepatocytes at the same time as methylglyoxal, had a non-significant decrease in cell death ($p=0.545$) at 120 minutes. Premixing each compound with methylglyoxal resulted in better protection, as noted by less cell death when compared to adding the catechins at the same time as methylglyoxal, however, these differences were not significant at 120 minutes. After 180 minutes, all three compounds significantly protected against cell death, when premixed or not premixed with methylglyoxal. Premixing the compounds with methylglyoxal resulted in better cytoprotection, however, only a significant difference was seen for the difference between premixing epicatechin with methylglyoxal versus not premixing, where the premix elicited protection by 40 % compared to not premixing (21 %) ($p=0.007$).

Table 3-5: Catechin, Epicatechin and EGCG prevent Methylglyoxal induced Cytotoxicity and Protein Carbonylation in GSH depleted Primary Rat Hepatocytes

Compounds Added	Percent Cytotoxicity (% trypan blue uptake)			Protein Carbonylation (nmol/10^6 cells)†	
	60 min	**120 min**	**180 min**	**45 min**	**120 min**
control – hepatocytes only	24 ± 3	28 ± 2	33 ± 2	7 ± 0.3	8 ± 0.6
+ methylglyoxal 10 mM	28 ± 1[b)]	32 ± 1[b)]	37 ± 1[b)]	497 ± 26.2[a) b)]	24 ± 1.3[a) b)]
GSH depleted hepatocytes					
+ methylglyoxal 3 mM	40 ± 4[a)]	54 ± 6[a)]	75 ± 8[a) b)]	411 ± 19.9[a)]	128 ± 6.2[a)]
+ catechin 3 mM	32 ± 2[b)]	41 ± 3[a) b)]	46 ± 3[a) b)]	64 ± 2.5[a) b)]	57 ± 2.2[a) b)]
+ catechin 3 mM^	29 ± 2[b)]	33 ± 2[b)]	37 ± 2[b)]	63 ± 2.7[a) b)]	44 ± 1.9[a) b)]
+ epicatechin 3 mM	35 ± 3[a)]	48 ± 4[a)]	59 ± 5[a) b)]	74 ± 2.7[a) b)]	63 ± 2.3[a) b]
+ epicatechin 3 mM^	35 ± 2[a)]	41 ± 2[a) b)]	45 ± 2[a) b)]	73 ± 2.6[a) b)]	61 ± 2.1[a) b)]
+ EGCG 3 mM	37 ± 4[a)]	42 ± 4[a) b)]	47 ± 5[a) b)]	94 ± 3.4[a) b)]	83 ± 3.0[a) b)]
+ EGCG 3 mM^	34 ± 5[a)]	35 ± 5[b)]	39 ± 6[a) b)]	79 ± 4.6[a) b)]	72 ± 4.2[a) b)]

Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37 °C in rotating round bottom flasks with 95 % O_2 and 5 % CO_2 in Krebs-Henseleit buffer (pH 7.4). † Protein carbonylation was measured as DNPH-derivatized samples as nmol/10^6 cells concentration (ε=2.2 x 10^4 M^{-1} cm^{-1}). GSH-depleted hepatocytes were obtained by preincubating hepatocytes with 200 µM 1-bromoheptane for 30 minutes, prior to the addition of other compounds. Means ± SE for three separate experiments are given.

^ Compound was premixed with methylglyoxal for 30 minutes prior to addition to hepatocytes

a) Significant as compared to control ($p < 0.05$)

b) Significant as compared to methylglyoxal 3 mM in GSH depleted hepatocytes($p < 0.05$)

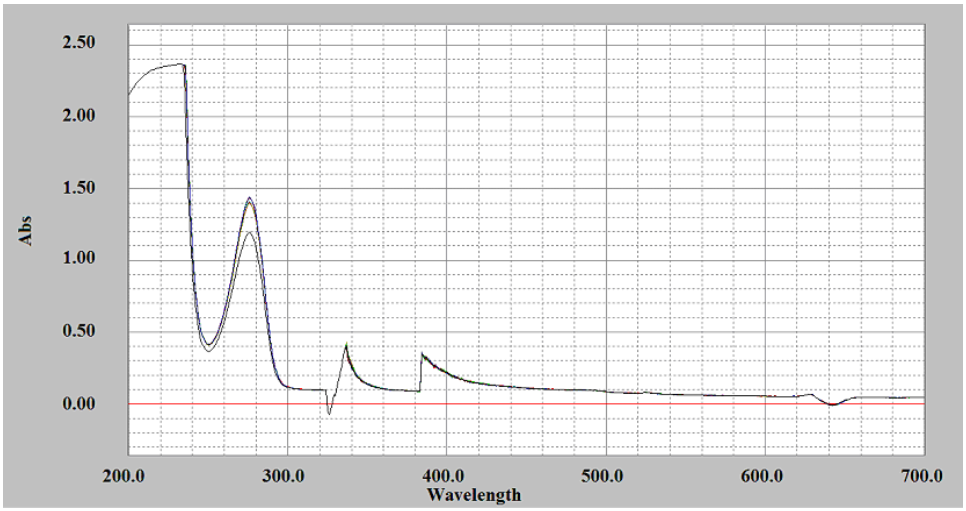
Summary: Pure catechin, epicatechin and EGCG all elicited significant protection against protein carbonylation in isolated hepatocytes, and there were no differences between adding the compound at the same time as or premixing with methylglyoxal, after 45 minutes. All three compounds significantly reduced carbonylation after 120 minutes, and when the different times of adding the compounds were compared, premixing catechin or EGCG were significantly different compared to adding catechin or EGCG at the same time as methylglyoxal ($p=0.002$; $p=0.010$ respectively for catechin and EGCG).

3.10 UV Spectrophotometer Scan of Catechin with Glyoxals

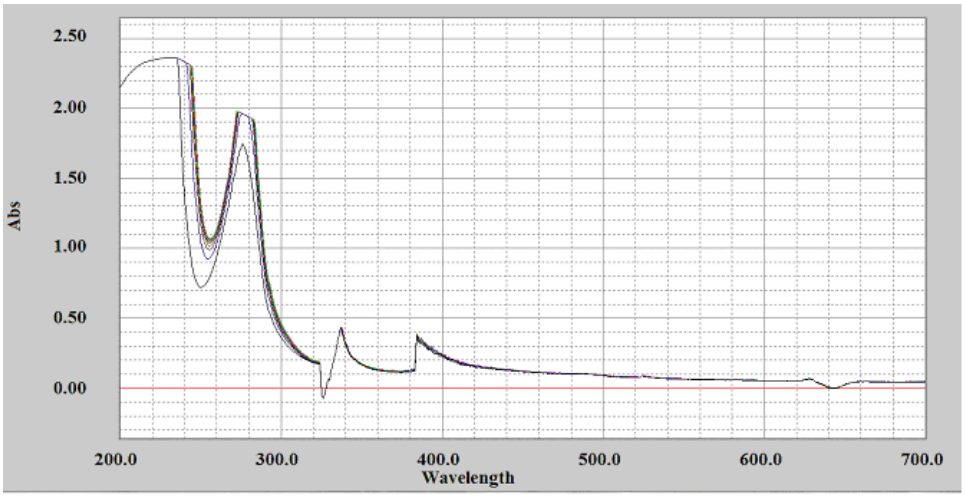
Totlani et al. used GC MS to show that epicatechin forms an adduct with methylglyoxal at 125 degrees centigrade (Totlani et al. 2006). However, adduct formation under physiological temperature and pH was not studied. Therefore, we investigated ultraviolet (UV) visual spectral scans of catechin mixed with methylglyoxal or glyoxal, measured at pH 7.4 and 37 degrees centigrade.

A scan of catechin alone every 6 minutes, over a 60 minute period, indicated a distinct absorbance peak at approximately 280 nm (Figure 3-7A), which was consistent with other literature data on the UV scan of catechin (Revilla et al. 1991). The absorbance of catechin steadily increased initially, and then remained at the same absorbance, with each scan over a 60 minute period. Addition of methylglyoxal showed a change in the peak after 60 minutes, such that the peak ranged over 275 to 285 nm (Figure 3-7B). Upon addition of glyoxal, the UV spectra of catechin remained unchanged over 60 minutes (Figure 3-7C).

A



B



C

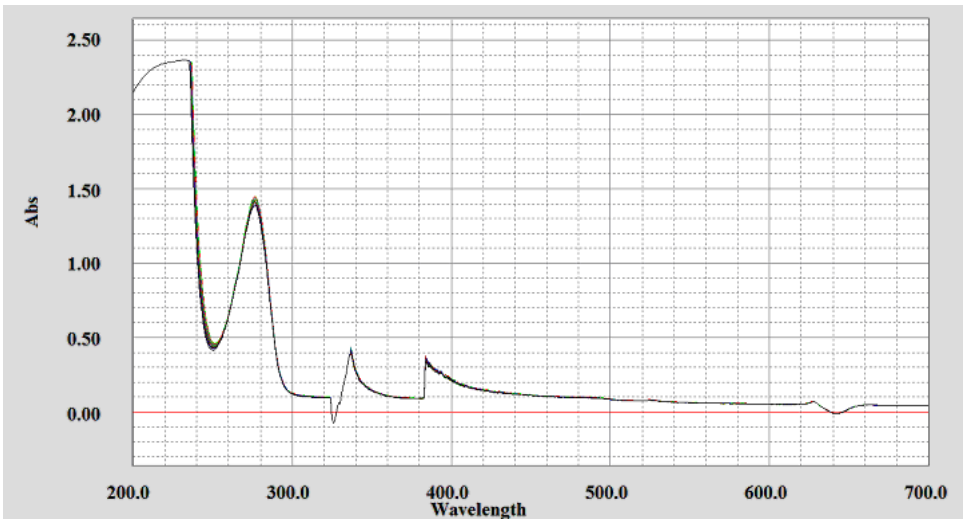


Figure 3-7. Ultraviolet Spectral Scans of Catechin with or without Glyoxals
UV visual spectral scans, ranging from 200 to 700 nm, were conducted every 6 minutes for 60 minutes, of A) catechin (300 µM) alone; B) catechin (300 µM) + methylglyoxal (15 mM); C) catechin (300 µM) + glyoxal (15 mM). All compounds were scanned in a quartz cuvette in 50 mM phosphate buffer (pH 7.4).

In summary, a change in the peak of the UV spectra data of catechin upon addition of methylglyoxal provides new evidence of a catechin-methylglyoxal adduct.

Chapter 5 – Discussion

Part I: Cytoprotection by Nut Extracts

There is a large body of evidence demonstrating the beneficial health effects of nut consumption in cardiovascular disease (Kris-Etherton et al. 2001), and more recently, in type 2 diabetes (Jiang et al. 2002). The positive outcomes observed across many studies prompted the FDA to issue a qualified health claim for nuts in 2003, recommending that the consumption of 1.5 ounces of nuts per day may reduce the risk of heart disease, in context of a healthy diet that is low in saturated fat and cholesterol (King et al. 2008). However, the molecular basis for the beneficial effect of nut consumption has not been fully elucidated. As oxidative stress is elevated in those afflicted with type 2 diabetes (Baynes et al. 1991), we sought to investigate the antioxidant activity of nuts in an *in vitro* model of oxidative stress.

We chose to investigate and compare extracts of walnuts and hazelnuts, as the consumption of these nuts in particular has been shown to elicit positive health effects such as enhancing plasma antioxidant potential and reducing cholesterol (for hazelnuts) (Durak et al. 1999) and an improved lipid profile (for walnuts) (Tapsell et al. 2004; Zibaeenezhad et al. 2005) in human clinical trials. Also, walnuts and hazelnuts have been reported to be among the nuts with higher total antioxidant capacities (TAC), when compared to the TACs of other commonly consumed nuts (Wu et al. 2004). Walnuts contained higher TACs compared to hazelnuts (135.41 versus 96.45 μmol of trolox

equivalent/gram). Walnuts also have been shown to contain double the content of total phenolics compared to hazelnuts (15.56 versus 8.35 mg of gallic acid equivalents/gram) (Wu et al. 2004). Furthermore, walnuts have been reported to have the highest total phenolic content among 10 different nut types examined, whereas hazelnuts had the fifth highest level of phenolic compounds (Kornsteiner et al. 2006). Extracts of hazelnut skin were recently examined where methanolic and acetone extracts of hazelnut skins were found to elicit antioxidant activity through several assays (Alasalvar et al. 2009). Furthermore, the methanolic crude extract was found to have higher total phenolics than the acetone crude extract (701 and 686 mg Catechin Equivalents (CE)/gram, respectively), while the total antioxidant activities were similar (636 and 633 mg Trolox Equivalents (TE)/gram, respectively). Overall, both walnuts and hazelnuts have been noted as good sources of antioxidants, attributed to the reducing power by phenolics compounds.

We chose three solvents (ethyl acetate, methanol or water) to prepare three extracts of each nut in order to investigate if the more hydrophilic extracts (using methanol or water) would elicit better cytoprotection against cell death compared to a more lipophilic extract (using ethyl acetate). The rationale for investigating different types of nut extracts was based on several observations. First, scientific literature has traditionally suggested that tocopherols (vitamin E) were the main antioxidant component of nuts, as this group of compounds has been largely focused on because nuts are a good source of tocopherols (vitamin E) (Kornsteiner et al. 2006). It is important to note that tocopherols would be located in the ethyl acetate extracts of walnuts or hazelnuts in the present study, as ethyl acetate dissolves relatively non-polar compounds such as tocopherol (Pereira et al. 2008; Miraliakbari and Shahidi 2008). However, more recent

evidence has shown that the antioxidant potential of nuts may be more likely to result from of their high content of polar compounds, (e.g. polyphenolic flavonoids). Many of these compounds are found in the skin/pellicle of the nut (Chen 2008; Wijeratne et al. 2006). For example, one study investigated the antioxidant activity of Sicilian pistachio extracts (either hydrophilic or lipophilic extracts) and found that the hydrophilic extract had a much higher antioxidant potential (by 50 fold) when compared to the lipophilic extract (Gentile et al. 2007). The study also investigated the effect of the hydrophilic pistachio extract in two biological models of lipid peroxidation. First, lipid oxidation was induced in bovine liver microsomal membranes by the hydrophilic azo-initiator AAPH [2,2'-Azobis(2-methylpropionamidine) dihydrochloride], and the hydrophilic pistachio extract (0.25-1 mg of pistachio nut) prevented TBARS formation in this system, in a dose dependent manner. The hydrophilic pistachio extract also prevented TBARS formation when Fe^{3+}/ascorbate were added to the system, however, only at the two higher concentrations used (0.5 and 1 mg of nut). Secondly, the extract was also able to increase resistance of human LDL to copper-induced LDL oxidation, by postponing the time course of conjugated diene lipid hydroperoxide formation during the copper induced oxidation of LDL, in a dose response manner. Taken altogether, the results from the pistachio study further confirmed the strong evidence of antioxidant and metal-chelating effects by a hydrophilic nut extract in several chemical and biological *in vitro* systems, attributed to the bioactive and phenolic content of the nut. Therefore, three different extracts for each nut were investigated in the present study.

Initially and before conducting any experiments with the nut extracts, we determined the ferric reducing/antioxidant power (FRAP) of each extract, and

hypothesized that the methanolic or the water (hydrophilic) extracts would have higher FRAP values than the ethyl acetate (more lipophilic) extracts. Our results showed that for both the walnut and hazelnut extracts, the order of antioxidant capacity (FRAP value), from highest to lowest was: methanolic > aqueous > ethyl acetate (Figure 3-1). In the FRAP assay, most nut extracts (200 μg/ml) were comparable in their reducing ability to several antioxidants that were tested, all at concentrations of 10 μM. For example, the walnut methanolic extract, which elicited the highest reducing ability of all the nut extracts, was comparable and similar to the reducing ability of 10 μM of ellagic acid and quercetin, both of which are regarded as powerful antioxidants (Priyadarsini et al. 2002; Festa et al. 2001; Soobrattee et al. 2005). However, due to limited data on bioavailability of antioxidant concentrations in human plasma, it is not possible to extrapolate the nut extract concentrations used in the present study to concentrations examined in *in vivo* human studies, especially considering that different antioxidants have unique metabolic pathways, and that metabolites of antioxidants differ in their relative activity compared to the parent compound (Frei and Higdon 2003).

Before beginning any experiments using isolated primary hepatocytes for our oxidative stress model, we wished to determine if the nut extracts would be able to protect against oxidative stress in a biological model, and if this protection occurred in a dose response manner. Measuring lipid peroxidation (assessed by the TBARS assay) in rat liver microsomes was used in this preliminary non-cell model of oxidative stress, as free radicals are known to cause oxidative damage, such as lipid peroxide formation (Glascott et al. 1992). To induce lipid peroxidation, an organic hydroperoxide, tertiary-butyl hydroperoxide (TBuOOH) was used, and this compound has been used to rapidly

induce lipid hydroperoxide formation in microsomes and hepatocytes (Mehta et al. 2008). Three different concentrations, ranging from 50-150 μg/ml, for each nut extract were tested for their ability to prevent lipid peroxidation in microsomes. Concentrations of nut extracts below 50 μg/ml did not elicit significant reductions in lipid peroxidation, thus 50 μg/ml was used as the minimum concentration for nut extracts in the microsomal lipid peroxidation assay. In this preliminary *in vitro* system, each extract elicited dose response protection against lipid peroxidation (Figures 3-2 and 3-3, Results section). Similar to our observation of a dose dependent protection by hazelnut or walnut extracts against lipid peroxidation in rat liver microsomes, Gentile et al. also observed an *in vitro* dose dependent protection by the hydrophilic pistachio extract in bovine liver microsomes (Gentile et al. 2007). The reduction in lipid peroxide formation in microsomes indicated that our hazelnut and walnut extracts elicited an antioxidant effect, and this protective effect may be contributed to the hydrogen donating ability by polyphenolic flavonoids (Figure 1-3) found in nuts and possibly the nut extracts.

For our main oxidative stress model, isolated primary hepatocytes were used because of the relevance of the liver in the processing and metabolism of nutrients in the digestive process. Tertiary-butyl hydroperoxide (TbuOOH) was used to induce oxidative stress. Cytotoxicity was determined by the trypan blue assay (assessing cell death). Also, lipid peroxidation was determined by the TBARS assay and reactive oxygen species (ROS) formation was measured by the DCFD method, and each nut extract was tested for its cytoprotective capacity, assessing cell death, lipid peroxidation and ROS formation. The polar extracts (including both, the methanolic and aqueous extracts) of hazelnuts and walnuts used in the present study elicited better cytoprotective abilities in isolated

primary hepatocytes than the non polar (ethyl acetate) extracts (or nut oils) (Table 3-2, Results) which indicated that antioxidants other than lipid soluble tocopherols, may be responsible for the antioxidant effect of nuts. However, for the hazelnut extracts, the water extract elicited better cytoprotection against cell death compared to the methanolic extract, whereas in the microsomal lipid peroxidation system, the methanolic extract elicited the best cytoprotection and the water extract was least protective. This observation may be due to the metabolism of cytoprotective components in the methanolic extract by hepatocyte glucuronidation or methylation metabolising enzymes. This renders compounds more water soluble but less cytoprotective (Donovan 2001). Conversely, it is also possible that metabolising enzymes in hepatocytes may be upregulated, thereby contributing to a cytoprotective effect against free radicals. This upregulation or downregulation of enzymes is dependent on time, as well as other possible differences that are likely to exist in rat hepatocytes between an *in vitro* and *in vivo* system. However, activation or inactivation of any enzymes was not determined in the present study, as the focus for the assays was on using the 'accelerated cytotoxicity mechanism screening method', a series of high throughput *in vitro* techniques to investigate cytotoxicity or cytoprotection outcomes of toxic compounds (O'Brien et al. 2004). Also, the upregulation or downregulation of enzymes likely requires much longer time periods (8-24 hours) to occur and detect, and hence may be determined in chronic toxicity studies.

Another study investigated the radical scavenging ability of methanolic extracts of peanut hulls (Yen & Duh 1994). The authors observed that the peanut methanolic extract, when added to 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH), H_2O_2 or a source of

superoxide or hydroxyl radicals, caused the disappearance of these various radicals, suggesting that the extract had *in vitro* radical scavenging ability. This study concluded that the peanut hull methanolic extract acted as both an oxygen scavenger and as an antioxidant.

It has been reported that walnuts (kernel with skin) contain approximately a 40 fold higher total antioxidant capacity compared to hazelnuts (kernel with skin) and most other nuts (Blomhoff, 2006). Furthermore, the ferric reducing/antioxidant power (FRAP) values for walnuts were found to be 32 times more effective in reducing ability compared to hazelnuts (Blomhoff, 2006). In our study, we found differences in the cytoprotective abilities between the walnut and hazelnut extracts, suggesting a relatively higher antioxidant effect of walnuts over hazelnuts, which elicited better protection against hepatocyte oxidative stress induced cell death. The more effective cytoprotective ability by walnut extracts is also reflected in our FRAP values, whereby the walnut methanolic extracts elicited significantly higher FRAP values compared to all hazelnut extracts and the walnut ethyl acetate extract. The walnut water extract also elicited significantly higher FRAP values compared to the hazelnut ethyl acetate and water extracts, but was similar to the hazelnut methanolic FRAP value. The differences in reducing ability by the extracts as determined by the FRAP assay, indicate the variability in antioxidant ability among different nuts and therefore, further strengthens recommendations for consuming mixed nuts, rather than a specific tree nut.

In one study, an analysis of a methanolic walnut extract revealed the presence of catechin, gallic acid, ellagic acid, coumaric acid and caffeine (Papoutsi et al. 2008). In a separate study, the polyphenols - quercetin, isorhamnetin, quercitrin, kaempferol-3-*O*-

rutinoside, and isorhamnetin 3-*O*-glucoside, were detected in ethanolic extracts of almond whole seed, brown skin, and green shell cover (Wijeratne et al. 2006). The authors also noted that the highest phenolic contents were found in the brown skin and green shell cover extracts (by 10 and 9 times higher, respectively) compared to the extract of the whole seed. Also, in a separate study, identified in methanolic extracts of almonds were 20 flavonoids (including epicatechin) and phenolic acids (Milbury et al. 2006). Interestingly, most of the phenolic acids found were in almond skins compared to almond kernel (blanched nut). On the other hand, catechin, epicatechin, procatechuic acid, *p*-hydroxy benzoic acid, vanillic acid and most (14 of the 20) of the other identified flavonoids were predominantly (>50 %) found in the blanched water (range 51 -83 %), compared to the almond skins and the blanched kernels (skinless), suggesting, that in our study, these phenolic compounds found in the walnut and hazelnut aqueous (water) extracts were likely responsible for the *in vitro* cytoprotection elicited by the water nut extracts.

Due to the variety of compounds found in nuts, it is likely that multiple cytoprotective mechanisms contribute to the overall protection against oxidative induced cytotoxicity in the hepatocyte model. One possible explanation for this protection may be due to the synergistic effect occurring between antioxidants, in which antioxidants recharge neighbouring antioxidants (Blomhoff 2006). An example of this is noted from one study which demonstrated that flavonoids from almond skins were found to act synergistically with vitamin C or vitamin E in protecting against LDL oxidation *in vitro* as well as enhancing resistance to Cu^{2+} induced oxidation of LDL *ex vivo* (Chen et al. 2005). Another study found that quercetin or epicatechin regenerated α-tocopherol, as

combinations of each flavonoid with α-tocopherol suppressed oxygen consumption more effectively when compared to each compound alone, in a methyl linoleate peroxidation system, thereby eliciting a co-antioxidant effect (Pedrielli and Skibsted 2002). Since whole hazelnut and walnuts were used in our studies, there may be synergistic effects occurring among the multiple bioactive compounds present in the extracts, and this may be partially responsible for cytoprotection against oxidative stress.

Another mechanism for cytoprotection by nut extracts may involve their metal chelating properties. One study found that the superoxide radical scavenging ability of the polyphenolics was a likely contributor to the antioxidative properties of almond ethanolic extracts (Siriwardhana et al. 2002). Almond extracts demonstrated excellent metal chelating abilities when tested against iron (Fe^{2+}) in a solution containing ferrous sulphate (Wijeratne et al. 2006). The ability of our extracts to prevent lipid peroxide formation in microsomes and in hepatocytes suggests that metal chelating activity of the nut extracts could be involved, as iron is known to generate lipid peroxides by forming hydroxyl ($OH^{\bullet}$) radicals or to generate alkoxyl radicals ($RO^{\bullet}$) from breaking down lipid peroxidation products (Lapidot et al. 2005).

Nuts also contain phytic acid (phytate), which is the predominant phosphorus storage compound of most nuts, seeds and cereal grains, consisting of approximately 1 – 7 % of the dry weight of these foods (Zhou and Erdman 1995). Phytic acid has been shown to chelate metal ions and is particularly effective against of iron, (Graf and Empson 1987) and in this way elicits an antioxidant effect. Phytates have been shown to be strong inhibitors of lipid peroxidation, and this has been hypothesized to be a result of effective blocking of hydroxyl, $OH^{\bullet}$ radical formation in an in vitro model of iron driven

OH• formation (Graf and Empson 1987; Rimbach and Pallauf 1998). Therefore, the phytic acid content of walnuts and hazelnuts may also be involved in the protective mechanism of the nut extracts in the present study, as phytic acid may act to reduce iron and other metal ions, thereby preventing ROS formation and lipid peroxidation.

A recent study examined the acute effects of nut consumption in healthy human subjects. The investigators found that both almonds and walnuts (in the form of smoothies) lead to significant increases in the plasma polyphenol concentration following the nut meals, with peak concentrations after 90 minutes (Torabien et al. 2009). More sustained effects on the plasma polyphenol level were noted for the walnut treatment compared to the almond treatment. Additionally, the susceptibility of plasma to lipid peroxidation decreased after 90 minutes on both nut meal treatments, but not on the control. It is interesting to note that these results on the decreasing susceptibility of plasma to lipid peroxidation in humans by almond or walnut consumption gave a similar result (protective effects of our hazelnut and walnut extracts) to what was observed in our *in vitro* studies. This suggests that a common mechanism for nuts is that they are a good source of bioactive compounds and antioxidants, and prevent lipid peroxidation likely by these constituents. It is important to note also that in both studies, the nuts were either blended (almond or walnut smoothie form in the study by Torabien) or ground into a fine powder (our walnut or hazelnut extracts), and therefore, differences in grinding or mastication of nuts may impact the bioaccessibility and the bioavailability of compounds in nuts.

Preventing or delaying atherosclerotic plaque formation may also be a mechanism by which nut consumption decreases the risk of cardiovascular disease in humans. The death of cells, specifically foam cells (derived from macrophages or smooth muscle cells), has been characterized as an early step in the formation of an atheroma (Ross 1999), ultimately leading to arteriosclerosis, a complication associated with type 2 diabetes. As hepatocyte cell death was prevented *in vitro* in the present study, as well as lipid peroxidation and ROS formation by nut extracts, another mechanism by which nuts are beneficial dietary components for type 2 diabetics may relate to cell survival mediated by antioxidants, possibly preventing atherosclerotic plaque formation in this manner.

Conclusion: Our *in vitro* experiments have shown a cytoprotective effect of crude nut extracts (particularly the methanolic extract) in an oxidative stress model against hydroperoxide induced lipid peroxidation, cytotoxicity and ROS formation. These results suggest that compounds found in the more water soluble extracts, made with methanol or water, elicit better cytoprotective abilities compared to the lipid soluble ethyl acetate extracts (oils), both in walnuts and hazelnuts.

Part II: Cytoprotection by Catechins against Dicarbonyls

In addition to oxidative stress, an elevated level of carbonyl stress has also been associated with type 2 diabetes, as a result of hyperglycemia. In fact, dicarbonyl compounds are known to be potent glycating agents, and have been estimated to be 200-

50,000-fold more reactive than glucose, despite their much lower physiological concentrations compared to glucose (Rabbani et al. 2008). Therefore, for the second part of our analyses, we focused on dicarbonyl compounds, specifically glyoxal and methylglyoxal, both of which have been implicated in the pathogenesis of diabetic complications as they are found in elevated levels in these patients (see Introduction). Catechins are a group of polyphenolic flavonoids (found in tree nuts, teas and other plants) which are essentially non toxic, and have demonstrated protective abilities against oxidative stress, and more recently, the ability to bind to and trap dicarbonyls. Evidence of the dicarbonyl trapping activity by catechins has been noted in some studies. For example, the trapping ability of methylglyoxal (MGO) by epicatechin in aqueous Maillard systems proposed a direct reaction of epicatechin with MGO (Totlani et al. 2006). In the same study, the chemical structures of two adducts were also identified, in which MGO covalently bound to the A ring of epicatechin (Figure 4-1). However the Maillard reaction reported in that study involved incubating 10 mM epicatechin with 10 mM MGO for 30 minutes at 125 °C, so it seemed unlikely that the reaction would occur under physiological temperature and pH.

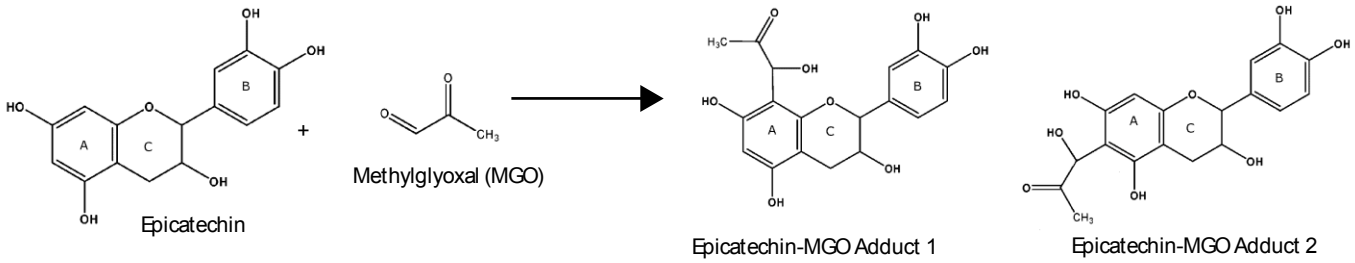


Figure 4-1: Proposed Epicatechin-Methylglyoxal (EC-MGO) adducts (Totlani et al. 2006)

Conversely, a different study examined tea polyphenols of green tea (EC, EGCG, EGC, ECG) and theaflavins in black tea under simulated physiological conditions (phosphate buffer, pH 7.4, 37 ° C), and found that these compounds effectively trapped methylglyoxal (MGO) by reducing the percentage of MGO in this system (Lo et al. 2006). The researchers further investigated EGCG for its trapping ability of MGO and glyoxal (GO), and identified novel EGCG-MGO and EGCG-GO adducts (Sang et al. 2007). The authors also compared the trapping ability of MGO by EGCG, to other known reactive carbonyl scavengers, such as the pharmaceutical agent, aminoguanidine (AG). In their system, MGO or GO (2 mM) were tested with or without EGCG, lysine, arginine or AG (all at 6 mM) and the remaining dicarbonyl (MGO or GO) left in the solution was observed over a 60 minute period, at a pH of 7.4 and 37 ° C. The authors concluded that EGCG was much more reactive with MGO or GO, compared to AG, whereas lysine or arginine were poor at carbonyl scavenging.

In the present study, bovine serum albumin (BSA) was used as the model protein for conducting preliminary cell-free analyses to compare catechins (specifically catechin, epicatechin, and EGCG) for their ability to prevent protein carbonylation induced by either glyoxal or methylglyoxal, under simulated physiological conditions (phosphate buffer, pH 7.4, 37 ° C), as other studies have only used buffer or aqueous systems (Sang et al. 2007; Lo et al. 2006; Totlani et al. 2006). Each catechin was added at the same time as glyoxal (GO) or methylglyoxal (MGO), at equimolar concentrations, to the BSA incubate. The three catechins significantly reduced protein carbonyl formation against MGO, however, only slightly reduced carbonyl formation by GO. This observation of a lower reactivity of catechins with GO may be attributed to the polymerization of the

hydrated GO monomer (the main form of aqueous GO) into dimers and trimers of GO. As a result, the equilibrium between monomers, dimers, trimers and free GO may explain the slower rate and lower reactivity of GO with catechins, and possibly other substrates that may bind to GO (Sang et al. 2007).

In the same system, and because Schiff base adducts are early stage products in the Maillard reaction (see Introduction), the next experiments were conducted to test if catechins could reverse early-stage AGE or Schiff base formation, induced by MGO or GO. Therefore, either MGO or GO were incubated with BSA for a 120 minute period to induce Schiff base formation, after which equimolar concentrations of catechin, or epicatechin or EGCG were added. Interestingly, a similar trend was seen as in the first experiments, where each catechin significantly reversed Schiff base formation against MGO; however, only catechin significantly decreased Schiff base formation induced by GO. As seen before, the effect of catechin against GO was much less reactive compared to the effect of catechin against MGO, with respect to protein carbonyl/Schiff base formation.

Finally, in the last set of cell-free experiments on serum albumin, catechin was chosen as the compound to further investigate as it displayed the most effective results in decreasing protein carbonylation, and decreasing Schiff base formation, compared to epicatechin or EGCG. Due to the known dicarbonyl trapping activity of catechin, the compound was premixed with either MGO or GO for a 30 minute period, after which it was added to the BSA-buffer system. The purpose of this experiment was to test if premixing catechin with either dicarbonyl would reduce protein carbonyl formation, and therefore would suggest reactivity of catechin with methylglyoxal and glyoxal as a

mechanism for reducing carbonylation, prior to investigating these compounds in hepatocytes. Premixing catechin with either dicarbonyl significantly reduced protein carbonylation at all time points (0, 30 and 120 minutes). The results were particularly evident very early at 0 minutes, where catechin reduced carbonylation by approximately 43 % and 54 % against GO or MGO induced carbonylation, respectively. In the other experiments, when premixing catechin with dicarbonyls was not done, the reductions in protein carbonylation by catechin were not significant or greatly reduced for glyoxal, and hence, suggesting that the catechin required time in order to bind with glyoxal and form an adduct in this system. With respect to MGO, catechin showed significant effects at reducing protein carbonyl formation when not premixed (31 % reduction). However, premixing catechin with MGO was much more effective as protein carbonylation was reduced by 54 %, 52 % and 80 % at 0 minutes, 30 and 120 minutes, respectively. The premixing of catechin with either dicarbonyl, continued to reduce carbonylation at 2 hours mostly for the MGO results (54 % at 0 min to 80 % at 120 min), while less for GO (43 % at 0 min to 47 % at 120 min) , suggesting that catechin required more time in order to further protect against carbonylation against MGO. Overall, these results indicate that premixing catechin with dicarbonyls allows catechin to trap GO and MGO and hence prevents these dicarbonyls from carbonylating proteins, using serum albumin.

The results on the BSA carbonylation experiments with catechins, along with others studies, indicated that the reactivity of catechins with dicarbonyls was able to occur under physiological temperature and pH, and using serum albumin (BSA) as a model protein. However, we have shown that catechins can also effectively reverse carbonylation when the dicarbonyls have had a substantial amount of time (120 minutes)

to induce protein carbonylation in BSA. Interestingly, we have also shown that actually premixing catechin with a dicarbonyl (MGO or GO) for a period of time could significantly reduce protein carbonylation immediately (0 minutes), hence, further strengthening the evidence for the binding capacity of catechin to the highly reactive dicarbonyls, glyoxal or methylglyoxal.

Isolated primary hepatocytes were used in the next investigations, as well as the same dicarbonyls (GO or MGO) to induce cell death and protein carbonylation. Initially, catechin was tested and added to the hepatocyte incubate at different times, against glyoxal induced cytotoxicity. Initially, the concentration of catechin used was twice as high as the glyoxal concentration (10 mM versus 5 mM). Catechin was added either to the incubate at 1) the same time as GO, or 2) 5 minutes after GO, or 3) 30 minutes after GO, or 4) it was premixed with GO for 30 minutes and then added to the incubate (Table 3-3, Results section).The premix of catechin with GO was most effective at preventing cell death (59 % cytotoxicity) when compared to adding catechin at the same time (66 % cytotoxicity) after 180 minutes. Adding catechin 5 or 30 minutes after GO had incubated with hepatocytes, was less effective, but nonetheless, still provided some protection against cell death (25 and 9 % protection against cell death, respectively). For the hepatocyte protein carbonylation results, the addition of catechin at the same time as GO decreased protein carbonylation most effectively (after 60 minutes), after which the premix provided the second best results. Interestingly, and similarly to the BSA results, catechin was able to reverse protein carbonylation, significantly when added 5 or 30

minutes after glyoxal, suggesting that the reduction in protein carbonylation may be one of the mechanisms by which catechin prevented cell death.

Next, catechin, epicatechin, and EGCG were tested for their ability to prevent cell death and protein carbonylation induced by GO, and a premix of each catechin with GO was also investigated. For each compound, the premix elicited better protection against cell death than adding the compound at the same time as GO, however, was only significant for epicatechin, after 180 minutes. Furthermore, the same trend was seen in the protein carbonylation results, where the premix of each catechin elicited a greater decrease in protein carbonyl formation than not premixing, however, was only significant for catechin ($p<0.001$) and EGCG ($p=0.037$). Epicatechin was highly effective with or without premixing it with glyoxal. A lower concentration of EGCG was used, and resulted in the best cytoprotective effects against glyoxal induce cell death and protein carbonylation, compared to catechin or epicatechin, although, the premix of each compound (catechin or epicatechin) with glyoxal, resulted in similar protein carbonyl concentrations after 120 minutes (Table 3-4) when compared to the premixed EGCG ($p=0.263$ and $p=0.438$, respectively). The reductions in protein carbonylation may likely be due to adducts formed by the catechins with glyoxal, thereby preventing glyoxal from causing its cytotoxic effects (through oxidative and carbonyl stress), and may be one mechanism to explain the cytoprotection elicited by these compounds in hepatocytes. EGCG, another catechin, is a major flavonoid of green tea and was reported to form mono-glyoxal-EGCG and di-glyoxal-EGCG or the equivalent methylglyoxal-EGCG adducts when EGCG was incubated with glyoxal or methylglyoxal respectively at pH 7.4 (Sang et al. 2007). Furthermore, EGCG was not previously tested for its effectiveness in

preventing glyoxal cell death or protein carbonylation in cells. Recently, several carbonyl scavenging drugs as well as some antioxidants have been previously investigated in our lab, against glyoxal induced cell death in hepatocytes (Mehta et al. 2009). The percent protection elicited by epicatechin (2.5 mM) (42 %) and EGCG (1 mM) (42 %) (when premixed with glyoxal) against glyoxal induced cell death in the present study was found to be more effective compared to: hydralazine (18-39 %), metformin (0 - 27 %), cysteine (8-24 %), N-acetylcysteine (NAC) (0-26 %), 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL) (18 %), Trolox (water soluble derivative of vitamin E) (38 %), and butylated hydroxyanisole (BHA) (17%), at the 180 minute time point (Mehta et al. 2009). Many of these compounds were tested in concentrations ranging from 1 -10 mM, depending on the compound, and only the much higher concentrations of other compounds (e.g. 5 mM aminoguanidine, 50 mM mannitol, 5 mM penicillamine, and 3 and 10 mM of pyrixodamine) were more effective at preventing glyoxal induced cell death compared to 2.5 mM of epicatechin and 1mM of EGCG, indicating that the efficacy of natural occurring flavonoids are comparable to known dicarbonyl trapping agents.

Catechin, epicatechin and EGCG were also examined in glutathione (GSH)-depleted hepatocytes. Methylglyoxal on its own is not toxic to hepatocytes (Shangari et al. 2006), as it is effectively and quickly metabolized by the GSH-dependent glyoxalase I (GLO I) system (Thornalley 1993; Vander Jagt & Hunsaker 2003). Glyoxalase 1 (GLO I) is a GSH-dependent enzyme responsible for catalyzing the metabolism of reactive dicarbonyls (Rabbani et al. 2008), hence preventing these dicarbonyls from glycating proteins. Therefore, once hepatocytes were depleted of their GSH using 1-bromoheptane,

MGO became toxic to hepatocytes, as noted by an increase in cell death. In this system, catechin, epicatechin and EGCG partially prevented cell death induced by MGO and were more effective when premixed with MGO, compared to not premixing; however, this was only significant for epicatechin, and was not significant for catechin or EGCG. Catechin and EGCG had similar protective abilities against cell death, whereas epicatechin was slightly less effective. Furthermore, all three compounds effectively reduced protein carbonylation, and only catechin and EGCG were significantly more effective when premixed compared to not premixing (catechin: $p=0.002$; EGCG: $p=0.010$). It is important to note that over time, the protein carbonylation levels decreased in the positive control. This may be due to continuous action or upregulation of the GLO I system (and therefore an increase in GSH production) or possibly other enzymes which may be metabolising MGO (i.e. oxidoreductases such as alpha-keto aldehyde dehydrogenase) (Vander Jagt & Hunsaker 2003) or possibly metabolising the MGO-catechin adducts.

A previous study by Totlani et al. had used GC MS evidence to show methylglyoxal-epicatechin adduct formation. A change in the UV spectra of catechin (i.e. shift or change in the absorbance peak) shown in the present study may be suggestive of the formation of an adduct, upon the addition of compounds. For example, a change in the UV spectra of catechin upon the addition of aluminum 3^{+} ions, suggested that the catechol moiety of catechin formed complexes with these ions *in vitro* (Tang et al. 2004). Methylglyoxal was found to alter the absorbance peak of catechin in the present study, suggesting that a methylglyoxal-catechin adduct was formed, whereas the addition of glyoxal to catechin resulted in no change to the absorbance peak of catechin, indicating

this adduct did not have a UV spectral absorbance. Interestingly, the change in the UV spectra of catechin by methylglyoxal indicates its reactivity with catechin, and this is consistent with our observations in BSA experiments, where catechin was found to prevent carbonylation by methylglyoxal much more effectively than glyoxal, which was much less reactive with catechin.

We have shown that catechin, epicatechin and EGCG protect cells from the cytotoxic effects of dicarbonyls likely by trapping dicarbonyls or by reversing dicarbonyl induced Schiff base formation (early stage glycation) when added to serum albumin after the dicarbonyl. Our lab has previously shown that aminoguanidine also prevents glyoxal induced cytotoxicity in primary hepatocytes by trapping glyoxal (Shangari et al. 2006; Mehta et al. 2008). It is important to note that adducts formed between the catechins and MGO or GO, may undergo further metabolism by drug metabolising enzymes in hepatocytes. Furthermore, the adducts could also undergo oxidation/reduction catalyzed by oxidoreductases in hepatocytes. Very limited data on catechin metabolism and bioavailability exists. However, one study showed that epicatechin was glucoronidated and to a lesser extent underwent sulfation in rat liver enzymes in cytosol (Vaidyanathan et al. 2002). Despite the limited data on catechin metabolism and on protective mechanisms *in vivo*, our results in BSA and in hepatocytes indicate that catechin, epicatechin and EGCG effectively prevented and reversed protein carbonyl formation, as well as prevented dicarbonyl induced cell death, which has not yet been shown. Perhaps further studies on *in vivo* animal models to measure plasma or urine levels of previously

characterized MGO-catechin adducts may be conducted, and would further determine the role of catechins in the prevention of early-stage glycation products.

In conclusion, epicatechin, catechin and EGCG were all found to prevent glyoxal or methylglyoxal induced cytotoxicity under physiological temperature (37 °C) and pH (pH 7.4), at concentrations lower than the dicarbonyls, in isolated primary rat hepatocytes. Furthermore, hepatocyte and BSA protein carbonylation induced by glyoxal or methylglyoxal was also partly prevented by all three catechin compounds. In most experiments, premixing the dicarbonyl with catechins resulted in better reductions against cytotoxicity, suggesting catechin-dicarbonyl adduct formation as a cytoprotective mechanism in hepatocytes.

Overall Conclusions

The effect of tree nuts on oxidative stress in type 2 diabetes remains to be elucidated. We have provided an *in vitro* evaluation of crude extracts made from whole walnuts and hazelnuts on their cytoprotection of a hepatocyte oxidative stress model of cell death. Surprisingly, the polar (methanolic, hydrophilic) extracts of the nuts elicited better protection compared to the non-polar (ethyl acetate) extracts; non-polar extracts/nut oils have been predominantly focused on in research as the non-polar vitamin E has thought to mainly contribute to the antioxidant capacity of nuts. Catechin, found in nuts and plants, was also found to be effective in protecting serum albumin or hepatocytes against methylglyoxal or glyoxal induced protein carbonyl formation or cell death respectively. Methylglyoxal and glyoxal are highly reactive aldehydes that are precursors of AGE products implicated in diseases associated with oxidative stress (such

as diabetes, aging and neurodegenerative diseases). To our knowledge, this is the first report of the cytoprotective activity of walnut and hazelnut extracts or catechins against cellular oxidative stress, protein carbonylation and cell death.

Future studies in humans with biomarkers should more conclusively prove and demonstrate that nuts in the diet decreases oxidative stress and protein carbonyl levels in type 2 diabetes.

Limitations of Research

Our *in vitro* model used to test nut extracts or catechins used isolated rat hepatocytes. It would be useful to investigate other cell systems that are susceptible to oxidative and carbonyl stress in diabetes patients, such as human cell lines (e.g. human kidney epithelial cell line; human hepatocyte culture; human endothelial cell line). However, there are some advantages to using primary hepatocytes in our studies such as the relevant role that the liver plays in the metabolism of xenobiotics and endogenous compounds, thus validating the use of hepatocytes. Additionally, our primary hepatocyte model is advantageous as it is an isolated system, whereby variables are easily controlled and markers are easily and quickly assayed.

Our studies used isolated primary hepatocytes for cytoprotection studies in acute experiments, in which the hepatocytes were viable for only a few hours (approximately 4 hours). Therefore, using cultured hepatocytes to explore cytoprotection in chronic toxicity studies would further indicate if nut extracts or catechins prevent toxicity in longer term studies, and the use of lower and physiologically relevant concentrations could also be incorporated in these studies. Interestingly, our lab has previously shown

that the molecular cytotoxic mechanisms for some xenobiotics studied *in vitro* in primary hepatocytes were similar to the hepatotoxic mechanisms in rats reported in *in vivo* experiments (O'Brien et al. 2004). Therefore, it is possible that our *in vitro* results may also occur in an *in vivo* animal model, however, this would have to be examined to know for certain that the *in vitro* hepatocyte results correlate with an *in vivo* hepatic situation, and this may be explored in future studies.

Investigating the effect of nuts or catechins in humans *in vivo* is the ultimate endpoint. However, because of ethical reasons, mechanistic studies (i.e. using cytotoxicity assays) are not conducted. Fortunately, it is possible to conduct clinical trials involving consumption of nuts and/or teas in human subjects, as these are dietary components which are already consumed, and markers of oxidative stress or carbonyl stress (compounds involved in the AGE pathway or AGEs) may be assayed in collected blood or urine samples. For example, diabetic subjects could be examined in a clinical feeding trial, and markers of oxidative stress in serum may be easily measured (i.e. oxidized LDL, protein thiols, malondialdehyde – TBARS assay). Additionally, carbonyl stress markers in serum, such as N^{ω}- carboxymethylarginine (Odani et al. 2001), N-carboxymethyllysine, and haemoglobin A1c (early stage AGE intermediate) may be measured. Serum or urinary isoprostanes (8-epi-prostaglandin F2a), which are formed *in vivo* as products of fatty acid peroxidation (mostly, peroxidation of arachidonic acid) are a reliable marker of oxidative stress *in vivo* (Minuz et al. 2006).

With respect to our protein carbonylation experiments in BSA, it is important to note that other model proteins may differ with respect to lysine and arginine content compared to BSA. It has been estimated that approximately 0.1-0.2 % of arginine and

lysine residues are glycated (Rabbani et al. 2008), and glycation may be elevated in some tissues where protein turnover occurs at a slower rate. Therefore, a range of proteins should be examined because of the differences in lysine and arginine contents of proteins, as well as their possible differences in the glycation reaction.

Chapter 6 – Future Perspectives

We wish to validate our research by using an *in vivo* animal model to determine if nut extracts or nut consumption or catechins elicit protective properties in preventing oxidative stress and carbonyl stress markers (involved in the AGE pathway) or AGE products in a diabetes rat model. A type I diabetes rat model may be induced by streptozotozin, a toxic compound that selectively targets and damages β-cells of the pancreas. However, because we are studying type 2 diabetes, as this is the most prevalent form of diabetes, a more suitable *in vivo* model would be to use the Goto-Kakizaki (GK) rat, which is a non-obese type 2 diabetes animal model, and is characterized by the lack of insulin response to glucose stimuli, yet having similar amounts of secretory granules of insulin as rats with a normal glucose tolerance test (Kimura et al. 1982). The GK rat is insulin resistant as early as 8 weeks (Bisbis et al. 1993). This model could be used to investigate the effect of nut consumption and green tea infusions (containing a good source of catechins) on carbonyl and oxidative stress markers, such as measuring plasma glyoxal, methylglyoxal, HbA1c, and AGE products, in an *in vivo* animal model of diabetes. One possible biomarker to measure could be glyceraldehyde-derived AGE products, which have been recently been investigated to be a marker of postprandial hyperglycemia in GK rats (Kitahara et al. 2008). Additionally, serum creatinine and blood urea nitrogen (BUN) may be measured as biological endpoints of toxicity and pathology of the kidney (nephropathy) in diabetic rats (Reyes et al. 1993).

Investigating other catechins (such as gallocatechin, epigallocatechin, epicatechin 3-gallate) for their possible dicarbonyl trapping ability against dicarbonyls in cell and cell-free models, and identifying new adducts may also be further explored.

It would also be of interest to assess albumin carbonylation and lipid peroxides in human plasma from a clinical trial that is currently being conducted at the Clinical Nutrition and Risk Factor Modification Centre, at St. Michael's Hospital in Toronto (Clinical Trials.gov). In a randomized clinical trial of type 2 diabetics, participants were randomized to either consume: mixed tree nuts (test group) or whole wheat muffins (control group) for a 3 month period as supplements to their normal diets. The test (tree nut) group versus the control (muffin) group could be compared for

a) plasma inhibition of rat liver microsome lipid peroxidation to test the ability to protect against oxidative stress (assessed by the TBARS assay)

b) plasma inhibition of albumin AGE formation (using the DNPH method for assessing early stage protection against a) protein carbonylation induced by reactive dicarbonyls and b) metal induced protein oxidation)

c) *in vivo* glycemic control and oxidative stress (assessed by HbA1c, fasting glucose, serum fructosamine, isoprostane output, C-reactive protein, oxidized LDL, plasma total antioxidant capacity (TAC), TBARS)

These types of studies would more clearly determine a real life *in vivo* effect of nut consumption on decreasing markers of oxidative stress in humans with type 2 diabetes. Such studies may lead to a dietary approach for prevention and therapy of diabetes by lessening oxidative and carbonyl stress, both of which are elevated in type 2 diabetic

patients. In this way, damage from glycation and oxidative stress may be minimized and hindered, by bioactive dietary components and may complement existing therapy for treatment of diabetes complications in humans.

References

Abou-Seif,MA & Youssef AA. Evaluation of some biochemical changes in diabetic patients. *Clin. Chim. Acta* **346**, 161-170 (2004).

Ahmed N & Thornalley PJ. Advanced glycation endproducts: what is their relevance to diabetic complications? *Diabetes Obes. Metab.* **9**, 233-245 (2007).

Alasalvar C, Shahidi F, Liyanapathirana CM, & Ohshima T. Turkish Tombul hazelnut (Corylus avellana L.). 1. Compositional characteristics. *J. Agric. Food Chem.* **51**, 3790-3796 (2003).

Alasalvar C, Karamac M, Kosinska A, Rybarczyk A, Shahidi F, & Amarowicz R. Antioxidant Activity of Hazelnut Skin Phenolics. *J. Agric. Food Chem.* **57**, 4645-4650 (2009).

Aldini G, Dalle-Donne I, Facino RM, Milzani A, & Carini M. Intervention strategies to inhibit protein carbonylation by lipoxidation-derived reactive carbonyls. *Med. Res. Rev.* **27**, 817-868 (2007).

Aron PM & Kennedy JA. Flavan-3-ols: Nature, occurrence and biological activity. *Mol. Nutr. Food Res.* **52**, 79-104 (2008).

Banach MS, Dong Q, & O'Brien PJ. Hepatocyte cytotoxicity induced by hydroperoxide (oxidative stress model) or glyoxal (carbonylation model): prevention by bioactive nut extracts or catechins. *Chem. Biol. Interact.* **178(1-3)**, 324-331 (2009).

Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes* **40**, 405-412 (1991).

Baynes JW & Thorpe SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* **48**, 1-9 (1999).

Beisswenger PJ, Howell SK, Nelson RG, Mauer M, & Szwergold BS. Alpha-oxoaldehyde metabolism and diabetic complications. *Biochem. Soc. Trans.* **31**, 1358-1363 (2003).

Beisswenger PJ, Howell SK, Touchette AD, Lal S, & Szwergold BS. Metformin reduces systemic methylglyoxal levels in type 2 diabetes. *Diabetes* **48**, 198-202 (1999).

Benzie IFF & Strain JJ. Ferric reducing ability of plasma (FRAP) as a measure of antioxidant power. *Anal. Biochem.* **239**, 70-76 (1996).

Bisbis S, Bailbe D, Tormo MA, Picarel-Blanchot F, Derouet M, Simon J, & Portha B. Insulin resistance in the GK rat: decreased receptor number but normal kinase activity in liver. *Am. J. Physiol.* **265**, E807-E813 (1993).

Blomhoff R, Carlsen MH, Andersen LF, & Jacobs DR, Jr. Health benefits of nuts: potential role of antioxidants. *Br. J. Nutr.* **96 Suppl 2**, S52-S60 (2006).

Bolen S, Feldman L, Vassy J, Wilson L, Yeh HC, Marinopoulos S, Wiley C, Selvin E, Wilson R, Bass EB, & Brancati FL. Systematic review: comparative effectiveness and safety of oral medications for type 2 diabetes mellitus. *Ann. Intern. Med.* **147**, 386-399 (2007).

Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813-820 (2001).
Brownlee M & Hirsch IB. Glycemic variability: a hemoglobin A1c-independent risk factor for diabetic complications. *JAMA*. **295(14)**, 1707-1708 (2006).

Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* **54**, 1615-1625 (2005).

Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813-820 (2001).

Cabrera C, Artacho R, & Gimenez R. Beneficial effects of green tea: a review. *J. Am. Coll. Nutr.* **25(2)**, 79-99 (2006).

Chen CY, Milbury PE, Lapsley K, & Blumberg JB. Flavonoids from almond skins are bioavailable and act synergistically with vitamins C and E to enhance hamster and human LDL resistance to oxidation. *J. Nutr.* **135**, 1366-1373 (2005).

Chen CY & Blumberg JB. In vitro activity of almond skin polyphenols for scavenging free radicals and inducing quinone reductase. *J. Agric. Food Chem.* **56**, 4427-4434 (2008).

Chung FL, Schwartz J, Herzog CR, &Yang YM. Tea and cancer prevention: studies in animals and humans. *J. Nutr*. **133 (10)**, 3268S-3274S (2003).

Clinical Trials.gov Effect of Nuts vs. a Wheat Bran Muffin in Type 2 Diabetes [accessed June 2009]
http://clinicaltrials.gov/ct2/show/NCT00410722?term=Jenkins+nut+muffin&rank=1

Crews C, Hough P, Godward J, Brereton P, Lees M, Guiet S, & Winkelmann W. Study of the main constituents of some authentic walnut oils. *J. Agric. Food Chem.* **53**, 4853-4860 (2005).

Crews C, Hough P, Godward J, Brereton P, Lees M, Guiet S, & Winkelmann W. Study of the main constituents of some authentic hazelnut oils. *J. Agric. Food Chem.* **53**, 4843-4852 (2005).

Dallner G. Isolation of microsomal subfractions by use of density gradients. *Methods Enzymol.* **52**, 71-82 (1978).

Standards of medical care in diabetes—2008, *Diabetes Care*, **31 Suppl 1**, S12–S54 (2008)

Donovan JL, Crespy V, Manach C, Morand C, Besson C, Scalbert A, & Remesy C. Catechin is metabolized by both the small intestine and liver of rats. *J. Nutr.* **131**, 1753-1757 (2001).

Drinkine J, Glories Y, Saucier C. (+)-Catechin-aldehyde condensations: Competition between acetaldehyde and glyoxylic acid. *J. Agric. Food Chem.* **53 (19)**, 7552-7558 (2005).

Durak I, Koksal I, Kacmaz M, Buyukkocak S, Cimen BM, & Ozturk HS. Hazelnut supplementation enhances plasma antioxidant potential and lowers plasma cholesterol levels. *Clin. Chim. Acta* **284**, 113-115 (1999).

Dyer DG, Dunn JA, Thorpe SR, Bailie KE, Lyons TJ, McCance DR, Baynes JW. Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J. Clin. Invest.* **91**, 2463-2469 (1993).

Fang Y, Yang S, Wu G. Free radicals, antioxidants and nutrition. *Nutrition* **18**, 872-879 (2002).

Festa F, Aglitti T, Duranti G, Ricordy R, Perticone P & Cozzi R. Strong antioxidant activity of ellagic acid in mammalian cells in vitro revealed by the comet assay. *Anticancer Res.* **21(6A)**, 3903-3908 (2001).

Finkel T & Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247 (2000).

Franz MJ, Bantle JP, Beebe CA, Brunzell JD, Chiasson JL, Garg A, Holzmeister LA, Hoogwerf B, Mayer-Davis E, Mooradian AD, Purnell JQ, & Wheeler M. Evidence-based nutrition principles and recommendations for the treatment and prevention of diabetes and related complications. *Diabetes Care* **25**, 148-198 (2002).

Frei B & Higdon JV. Antioxidant Activity of Tea Polyphenols In Vivo: Evidence from Animal Studies. *J. Nutr.* **133**, 3275S-284S (2003).

Fukuda T, Ito H, & Yoshida T. Antioxidative Polyphenols from walnuts (*Juglans Regia* L.) *Phytochemistry* **63**, 795-801 (2003).

Garg ML, Blake RJ, & Wills RB. Macadamia nut consumption lowers plasma total and LDL cholesterol levels in hypercholesterolemic men. *J. Nutr.* **133**, 1060-1063 (2003)

Gebauer SK, West SG, Kay CD, Alaupovic P, Bagshaw D, & Kris-Etherton PM. Effects of pistachios on cardiovascular disease risk factors and potential mechanisms of action: a dose-response study. *Am J Clin Nutr.* **88(3)**, 651-659 (2008).

Gentile C, Tesoriere L, Butera D, Fazzari M, Monastero M, Allegra M, & Livrea MA. Antioxidant activity of Sicilian pistachio (Pistacia vera L. var. Bronte) nut extract and its bioactive components. *J. Agric. Food Chem.* **55**, 643-648 (2007).

Glascott PA Jr, Gilfor E, & Farber JL. Effects of Vitamin E on the killing of cultured hepatocytes by tert-butyl hydroperoxide. *Mol. Pharmacol.* **41(6)**, 1155-1162 (1992).

Gomez-Lechon MJ, O'Connor E, Castell JV, & Jover R. Sensitive markers used to identify compounds that trigger apoptosis in cultured hepatocytes. *Toxicol. Sci.* **65**, 299-308 (2002).

Graf E & Empson KL. Phytic Acid – A Natural Antioxidant. *J. Biol. Chem.* **262(24)**, 11647-11650 (1987).

Griel AE, Cao Y, Bagshaw DD, Cifelli AM, Holub B, & Kris-Etherton PM. A macadamia nut-rich diet reduces total and LDL-cholesterol in mildly hypercholesterolemic men and women. *J Nutr.* **138(4)**, 761-767 (2008).

Gugliucci A. Glycation as the glucose link to diabetic complications. *J. Am. Osteopath. Assoc.* **100**, 621-634 (2000).

Halliwell B. Free radicals and antioxidants: a personal review. *Nutr. Rev.* **52**, 253-265 (1994).

Halliwell B. Antioxidants in human health and disease. Annu. *Rev. Nutr.* **16**, 33-50 (1996).

Halvorsen BL, Holte K, Myhrstad MC, Barikmo I, Hvattum E, Remberg SF, Wold AB, Haffner K, Baugerod H, Andersen LF, Moskaug O, Jacobs DR, Jr, & Blomhoff R. A systematic screening of total antioxidants in dietary plants. *J. Nutr.* **132**, 461-471 (2002).

Han Y, Randell E, Vasdev S, Gill V, Gadag V, Newhook LA, Grant M, & Hagerty D. Plasma methylglyoxal and glyoxal are elevated and related to early membrane alteration in young, complication-free patients with Type 1 diabetes. *Mol. Cell Biochem.* **305**, 123-131 (2007).

Harnley JM, Doherty RF, Beecher GR, Holden JM, Haytowitz DB, Bhagwat S, & Gebhardt S. Flavonoid Content of U.S. Fruits, Vegetables and Nuts. *J. Agric. Food Chem.* **54**, 9966-9977 (2006).

Hartley DP, Kroll DJ, & Petersen DR. Prooxidant-initiated lipid peroxidation in isolated rat hepatocytes: detection of 4-hydroxynonenal- and malondialdehyde-protein adducts. *Chem. Res. Toxicol.* **10**, 895-905 (1997).

Henle T, Deppisch R, & Ritz E. The Maillard reaction – from food chemistry to uraemia research. *Nephrol. Dial. Transplant.* **11(9)**, 1718-1722 (1996).

Hirsch IB & Brownlee M. Should minimal blood glucose variability become the gold standard of glycemic control? *J. Diabetes Complications*. **19(3)**, 178-181 (2005).

Hu FB & Stampfer MJ. Nut consumption and risk of coronary heart disease: a review of epidemiologic evidence. *Curr. Atheroscler. Rep.* **1**, 204-209 (1999).

Hu FB. Sedentary lifestyle and risk of obesity and type 2 diabetes. *Lipids*. **38(2)**, 103-108 (2003).

Hwang JM, Wang CJ, Chou FP, Tseng TH, Hsieh YS, Hsu JD, & Hu CY. Protective effect of baicalin on tert-butyl hydroperoxide-induced rat hepatotoxicity. *Arch. Toxicol.* **79**, 102-109 (2005).

Jambazian PR, Haddad E, Rajaram S, Tanzman J, & Sabate J. Almonds in the diet simultaneously improve plasma alpha-tocopherol concentrations and reduce plasma lipids. *J Am Diet Assoc.* **105(3)**, 449-454 (2005).

Jenkins DJ, Kendall CW, Marchie A, Parker TL, Connelly PW, Qian W, Haight JS, Faulkner D, Vidgen E, Lapsley KG, & Spiller GA. Dose response of almonds on coronary heart disease risk factors: blood lipids, oxidized low-density lipoproteins, lipoprotein(a), homocysteine, and pulmonary nitric oxide: a randomized, controlled, crossover trial. *Circulation* **106**, 1327-1332 (2002).

Jenkins DJ, Kendall CW, Josse AR, Salvatore S, Brighenti F, Augustin LS, Ellis PR, Vidgen E, & Rao AV. Almonds decrease postprandial glycemia, insulinemia, and oxidative damage in healthy individuals. *J Nutr.* **136**, 2987-2992 (2006).

Jiang R, Manson JE, Stampfer MJ, Liu S, Willett WC, & Hu FB. Nut and peanut butter consumption and risk of type 2 diabetes in women. *JAMA* **288**, 2554-2560 (2002).

Kesavulu MM, Rao BK, Giri R, Vijaya J, Subramanyam G, & Apparao C. Lipid peroxidation and antioxidant enzyme status in Type 2 diabetics with coronary heart disease. *Diabetes Res. Clin. Pract.* **53**, 33-39 (2001).

Khan S & O'Brien PJ. 1-bromoalkanes as new potent nontoxic glutathione depletors in isolated rat hepatocytes. Biochem. Biophys. *Res. Commun.* **179(1)**, 436-441 (1991).

Kimura K, Toyota T, Kakizaki M, Kudo M, Takebe K, & Goto Y. Impaired insulin secretion in the spontaneous diabetes rats. *Tohoku J. Exp. Med.* **137**, 453-459 (1982).

King JC, Blumberg J, Ingwersen L, Jenab M, & Tucker KL. Tree nuts and peanuts as components of a healthy diet. *J. Nutr.* **138**, 1736S-1740S (2008).

Kitahara Y, Takeuchi M, Miura K, Mine T, Matsui T, & Yamagishi S. Glyceraldehyde-derived advanced glycation end products (AGEs). A novel biomarker of postprandial hyperglycaemia in diabetic rats. *Clin. Exp. Med.* **8**, 175-177 (2008).

Kocyigit A, Koylu AA, & Keles H. Effects of pistachio nuts consumption on plasma lipid profile and oxidative status in healthy volunteers. *Nutr Metab Cardiovasc Dis.* **16(3)**, 202-209 (2006).

Kornsteiner M, Wagner K, Elmadfa I. Tocopherols and total phenolics in 10 different nut types. *Food Chem.* **98(2)**, 381-387 (2006).

Krentz AJ, Ferner RE, & Bailey CJ. Comparative tolerability profiles of oral antidiabetic agents. *Drug Saf* **11**, 223-241 (1994).

Krentz AJ & Bailey CJ. Oral antidiabetic agents: current role in type 2 diabetes mellitus. *Drugs* **65**, 385-411 (2005).

Kris-Etherton PM, Yu-Poth S, Sabate J, Ratcliffe HE, Zhao G, & Etherton TD. Nuts and their bioactive constituents: effects on serum lipids and other factors that affect disease risk. *Am. J. Clin. Nutr.* **70**, 504S-511S (1999).

Kris-Etherton PM, Zhao G, Binkowski AE, Coval SM, & Etherton TD. The effects of nuts on coronary heart disease risk. *Nutr. Rev.* **59(4)**, 103-111 (2001).

Lachman J, Sulc M, Faitova K, & Pivec V. Major factors influencing antioxidant contents and antioxidant activity in grapes and wine. *International Journal of Wine Research*. **1**, 101-121 (2009).

Lapidot T, Granit R, & Kanner J. Lipid peroxidation by "free" iron ions and myoglobin as affected by dietary antioxidants in simulated gastric fluids. *J. Agri. Food Chem.* **53**, 3383-3390 (2005).

Lapolla A, Flamini R, Dalla VA, Senesi A, Reitano R, Fedele D, Basso E, Seraglia R, & Traldi P. Glyoxal and methylglyoxal levels in diabetic patients: quantitative determination by a new GC/MS method. *Clin. Chem. Lab Med.* **41**, 1166-1173 (2003).

Lavedrine F, Ravel A, Poupard A & Alary J. Effect of geographic origin, variety and storage on tocopherol concentrations in walnuts by HPLC. *Food Chem*. **58**,135-140 (1997).

Lebovitz HE. Differentiating members of the thiazolidinedione class: a focus on safety. *Diabetes Metab Res. Rev.* **18 Suppl 2**, S23-S29 (2002).

Lemasters JJ, Qian T, Bradham CA, Brenner DA, Cascio WE, Trost LC, Nishimura Y, Nieminen AL & Herman B. Mitochondrial dysfunction in the pathogenesis in necrotic and apoptotic cell death. *J. Bioenerg. Biomembr.* **31(4)**, 305-319 (1999).

Li L, Tsao R, Yang R, Kramer JK, & Hernandez M. Fatty acid profiles, tocopherol contents, and antioxidant activities of heartnut (Juglans ailanthifolia Var. cordiformis) and Persian walnut (Juglans regia L.). *J. Agric. Food Chem.* **55**, 1164-1169 (2007).

Li L, Tsao R, Yang R, Liu C, Zhu H, & Young JC. Polyphenolic Profiles and Antioxidant Activities of Heartnut (*Juglans ailanthifolia* Var. *cordiformis*) and Persian Walnut (*Juglans regia* L.) *J. Agric. Food Chem.* **54(21)**, 8033-8040 (2006).

Lo CY, Li S, Tan D, Pan MH, Sang S, & Ho CT. Trapping reactions of reactive carbonyl species with tea polyphenols in simulated physiological conditions. *Mol. Nutr. Food. Res.* **50(12)**, 1118-1128 (2006).

Lokko P, Lartey A, Armar-Klemesu M, & Mattes RD. Regular peanut consumption improves plasma lipid levels in healthy Ghanaians. *Int. J. Food Sci. Nutr.* **58(3)**, 190-200 (2007).

Magalhaes LM, Segundo MA, Reis S, & Lima JLFC. Methodological aspects about in vitro evaluation of antioxidant properties. *Anal. Chim. Acta.* **613**, 1-19 (2008).

Maguire LS, O'Sullivan SM, Galvin K, O'Connor TP, & O'Brien NM. Fatty acid profile, tocopherol, squalene and phytosterol content of walnuts, almonds, peanuts, hazelnuts and the macadamia nut. *Int. J. Food Sci. Nutr.* **55**, 171-178 (2004).

Marchand S, de Revel G, & Bertrand A. Approaches to wine aroma: release of aroma compounds from reactions between cysteine and carbonyl compounds in wine. *J. Agric. Food Chem*. **48(10)**, 4890-4895 (2000).

McCance DR, Dyer DG, Dunn JA, Bailie KE, Thorpe SR, Baynes JW, & Lyons TJ. Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. *J. Clin. Invest.* **91**, 2470-2478 (1993).

Mehta R, Shangari N, & O'Brien PJ. Preventing cell death induced by carbonyl stress, oxidative stress or mitochondrial toxins with vitamin B anti-AGE agents. *Mol. Nutr. Food Res.* **52**, 379-385 (2008).

Mehta R, Wong L, & O'Brien PJ. Cytoprotective mechanisms of carbonyl scavenging drugs in isolated rat hepatocytes. *Chem. Biol. Interact*. **178(1-3)**,317-323 (2009).

Milbury PE, Chen CY, Dolnikowski GG, & Blumberg JB. Determination of flavonoids and phenolics and their distribution in almonds. *J. Agric. Food Chem.* **54**, 5027-5033 (2006).

Minuz P, Fava C, & Lechi A. Lipid peroxidation, isoprostanes and vascular damage. *Pharmacol. Rep.* **58**, 57S-68S (2006).

Miraliakbari H & Shahidi F. Oxidative stability of tree nut oils. *J. Agric. Food Chem.* **56**, 4751-4759 (2008).

Miyata T, Kurokawa K, & van Ypersele de Strihou C. Advanced Glycation and Lipoxidation End Products: Role of Reactive Carbonyl Compounds Generated during Carbohydrate and Lipid Metabolism. *J. Am. Soc. Nephrol.* **11**, 1744-1752 (2000).

Miyazaki Y, Kawano H, Yoshida T, Miyamoto S, Hokamaki J, Nagayoshi Y, Yamabe H, Nakamura H, Yodoi J, & Ogawa H. Pancreatic B-cell function is altered by oxidative stress induced by acute hyperglycaemia. *Diabet. Med.* **24**, 154-160 (2007).

Moldeus P, Hogberg J, & Orrenius S. Isolation and use of liver cells. *Methods Enzymol.* **52**, 60-71 (1978).

Monnier L, Mas E, Ginet C, Michel F, Villon L, Cristol JP, & Colette C. Activation of Oxidative Stress by Acute Glucose Fluctuations Compared with Sustained Chronic Hyperglycemia in Patients with Type 2 Diabetes. *JAMA*, **295**, 1681-1687 (2006).

Morgan WA & Clayshulte BJ. Pecans lower low-density lipoprotein cholesterol in people with normal lipid levels. *J. Am. Diet. Assoc.* **100**, 312-318 (2000).

Morrell CN. Reactive Oxygen Species – finding the right balance. *Circ. Res*. **103**, 571-572 (2008).

Mukuddem-Peterson J, Oosthuizen W, & Jerling JC. A systematic review of the effects of nuts on blood lipid profiles in humans. *J. Nutr.* **135**, 2082-2089 (2005).

Njoroge FG & Monnier VM. The chemistry of the Maillard reaction under physiological conditions: a review. *Prog. Clin. Biol. Res.* **304**, 85-91 (1989).

O'Brien PJ, Siraki AG, & Shangari N. Aldehyde sources, metabolism, molecular toxicity mechanisms, and possible effects on human health. *Crit Rev. Toxicol.* **35**, 609-662 (2005).

O'Brien PJ, Chan K, & Silber PM. Human and animal hepatocytes in vitro with extrapolation in vivo. *Chem. Biol. Interact.* **150**, 97-114 (2004).

Odani H, Iijima K, Nakata M, Miyata S, Kusunoki H, Yasuda Y, Hiki Y, Irie S, Maeda K, & Fujimoto D. Identification of N(omega)-carboxymethylarginine, a new advanced glycation endproduct in serum proteins of diabetic patients: possibility of a new marker of aging and diabetes. *Biochem. Biophys. Res. Commun*. **285(5)**, 1232-1236 (2001).

Papoutsi Z, Kassi E, Chinou I, Halabalaki M, Skaltsounis LA, & Moutsatsou P. Walnut extract (Juglans regia L.) and its component ellagic acid exhibit anti-inflammatory activity in human aorta endothelial cells and osteoblastic activity in the cell line KS483. *Br. J. Nutr.* **99**, 715-722 (2008).

Pedrielli P & Skibsted LH. Antioxidant synergy and regeneration effect of quercetin, (-)-epicatechin, and (+)-catechin on alpha-tocopherol in homogeneous solutions of peroxidating methyl linoleate. *J. Agric. Food Chem.* **50**, 7138-7144 (2002).

Pellegrini N, Serafini M, Salvatore S, Del Rio D, Bianchi M, & Brighenti F. Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different in vitro assays. *Mol. Nutr. Food Res.* **50**, 1030-1038 (2006).

Pennathur S & Heinecke JW. Mechanisms of oxidative stress in diabetes: implications for the pathogenesis of vascular disease and antioxidant therapy. *Front Biosci.* **9**, 565-574 (2004).

Peppa M, Uribarri J, & Vlassara H. Glucose, Advanced Glycation End Products, and Diabetes Complications: What Is New and What Works. *Clinical Diabetes*, **21(4)**, 186-187 (2003).

Pereira JA, Oliveira I, Sousa A, Fereira I.CFR, Bento A, & Estevinho L. Bioactive properties and chemical composition of six walnut (*Juglans Regia* L.) cultivars. *Food Chem. Toxicol.* **46**, 2103-2111 (2008).

Pratt DE & Miller EE. A Flavonoid Antioxidant in Spanish Peanuts (Arachia hypogoea). *J. Am. Oil Chem. Soc.* **61(6)**, 1064-1067 (1984).

Priyadarsini KI, Khopde SM, Kumar SS, & Mohan H. Free Radical Studies of Ellagic Acid, a Natural Phenolic Antioxidant. *J. Agric. Food Chem.* **50**, 2200-2206 (2002).

Rabbani N & Thornalley PJ. Dicarbonyls linked to damage in the powerhouse: glycation of mitochondrial proteins and oxidative stress. *Biochem. Soc. Trans*. **36** (Pt 5), 1045-1050 (2008).

Rahbar S & Figarola JL. Novel inhibitors of advanced glycation endproducts. *Arch. Biochem. Biophys.* **419**, 63-79 (2003).

Rajaram S, Burke K, Connell B, Myint T, & Sabaté J. A monounsaturated fatty acid-rich pecan-enriched diet favorably alters the serum lipid profile of healthy men and women. *J. Nutr*. **131(9)**, 2275-2279 (2001).

Revilla E, Bourzeix M, & Alonso E. Analysis of Catechins and Proanthocyanidins in Grape Seeds by HPLC with Photodiode Array Detection. *Chromatographia*. **31**, 465-468, (1991).

Reyes AA, Karl IE, Kissane J, & Klahr S. L-Arginine administration prevents glomerular hyperfiltration and decreases proteinuria in diabetic rats. *J. Am. Soc. Nephrol*. **4**,1039-1045, (1993).

Rimbach G & Pallauf J. Phytic Acid Inhibits Free Fadical Formation In Vitro but Does Not Affect Liver Oxidant or Antioxidant Status in Growing Rats. *J. Nutr*. **128(11)**, 1950-1955 (1998).

Rosengarten F. The Book of Edible Nuts. Dover Publications, NY (2004).

Rosca MG, Monnier VM, Szweda LI, & Weiss MF Alterations in renal mitochondrial respiration in response to the reactive oxoaldehyde methylglyoxal. *Am. J. Physiol Renal Physiol* **283**, F52-F59 (2002).

Sabate J, Fraser GE, Burke K, Knutsen SF, Bennett H, & Lindsted KD. Effects of Walnuts on Serum Lipid Levels and Blood Pressure in Normal Men. *N. Engl. J. Med.* **328(9)**, 603-607, (1993).

Sabate J, Ros E, & Salas-Salvado J. Nuts: nutrition and health outcomes. Preface. *Br. J. Nutr.* **96 Suppl 2**, S1-S2 (2006).

Sakuragawa A, Yoneno T, Inoue K, & Okutani T. Trace analysis of carbonyl compounds by liquid chromatography-mass spectrometry after collection as 2,4-dinitrophenylhydrazine derivatives. *J. Chromatogr. A.* **844(1-2)**, 403-408 (1999).

Sanders TH, McMichael RW Jr, & Hendrix KW. Occurrence of resveratrol in edible peanuts. *J. Agric. Food Chem.* **48(4)**, 1243-1246 (2000).

Sang S, Shao X, Bai N, Lo CY, Yang CS, & Ho CT. Tea polyphenol (-)-epigallocatechin-3-gallate: a new trapping agent of reactive dicarbonyl species. *Chem. Res. Toxicol.* **20**, 1862-1870 (2007).

Sano J, Inami S, Seimiya K, Ohba T, Sakai S, Takano T, Mizuno K. Effects of green tea intake on the development of coronary artery disease. *Circ. J.* **68 (7)**, 665-670 (2004).

Shahidi F, Alasalvar C, & Liyana-Pathirana CM. Antioxidant phytochemicals in hazelnut kernel (Corylus avellana L.) and hazelnut byproducts. *J. Agric. Food Chem.* **55**, 1212-1220 (2007).

Shangari N & O'Brien PJ. The cytotoxic mechanism of glyoxal involves oxidative stress. *Biochem. Pharmacol.* **68**, 1433-1442 (2004).

Shangari N, Chan TS, Popovic M, & O'Brien PJ. Glyoxal markedly compromises hepatocyte resistance to hydrogen peroxide. *Biochem. Pharmacol.* **71**, 1610-1618 (2006).

Shangari N, Poon R, & O'Brien PJ. Hepatocyte Methylglyoxal (MG) Resistance is overcome by inhibiting aldo-keto reductases (AKRs) and glyoxalase I (GLO I) catalyzed MG metabolism. *Enzymol. Mol. Biol. Carbonyl Metab*. **12**, 266-275 (2006).

Sheetz MJ. & King GL. Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *JAMA* **288**, 2579-2588 (2002).

Sheridan MJ, Cooper JN, Erario M, & Cheifetz CE. Pistachio nut consumption and serum lipid levels. *J. Am. Coll. Nutr*. **26(2)**, 141-148 (2007).

Shulze M & Hu FB. Primary Prevention of Diabetes: What Can be Done and How Much can be Prevented? *Annu. Rev. Public Health*. **26**, 445-467 (2005).

Schutte AE, Van Rooyen JM, Huisman HW, Mukuddem-Petersen J, Oosthuizen W, Hanekom SM, & Jerling JC. Modulation of baroreflex sensitivity by walnuts versus cashew nuts in subjects with metabolic syndrome. *Am. J. Hypertens*. **19(6)**, 629-636 (2006).

Singal P, Khaper N, Palace V, & Kumar D. The role of oxidative stress in the genesis of heart disease. *Cardiovasc. Res.* **40**, 426-432 (1998).

Siriwardhana SSKW & Shahidi F. Antiradical activity of extracts of almond and its by-products. *J. Am. Oil Chem. Soc.* **79(9)**, 903-908 (2002).

Smith MT, Thor H, Hartizell P, & Orrenius S. The measurement of lipid peroxidation in isolated hepatocytes. *Biochem. Pharmacol.* **31**, 19-26 (1982).

Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, & Bahorun T. Phenolics as potential antioxidant therapeutic agents: Mechanisms and actions. *Mutat. Res.* **579**, 200-213(2005).

Stoscheck CM. Quantitation of protein. *Methods Enzymol.* **182**, 50-68 (1990).

Sullivan KA & Feldman EL. New developments in diabetic neuropathy. *Curr. Opin. Neurol.* **18**, 586-590 (2005).

Tang DS, Shen SR, Chen X, Zhang YY, & Xu CY. Interaction of catechins with aluminum in vitro. *J. Zhejiang Univ. Sci.* **5(6)**, 668-675 (2004).

Tapsell LC, Gillen LJ, Patch CS, Batterham M, Owen A, Bare M, & Kennedy M. Including walnuts in a low-fat/modified-fat diet improves HDL cholesterol-to-total cholesterol ratios in patients with type 2 diabetes. *Diabetes Care* **27(12)**, 2777-2783 (2004).

Thomson CD, Chisholm A, McLachlan SA, & Campbell JM. Brazil nuts: an effective way to improve selenium status. *Am. J. Clin. Nutr*. **87(2)**, 379-384 (2008).

Thornalley PJ, Langborg A, & Minhas HS. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem. J.* **344 Pt 1**, 109-116 (1999).

Thornalley PJ. The glyoxalase system in health and disease. *Mol. Aspects Med.* **14(4)**, 287-371 (1993).

Traber MG & Atkinson J. Vitamin E, antioxidant and nothing more. *Free Radic. Biol. Med*. **43**, 4-15 (2007).

Torabien S, Haddad E, Rajaram S, Banta J, & Sabate J. Acute effect of nut consumption on plasma total polyphenols, antioxidant capacity and lipid peroxidation. *J. Hum. Nutr. Diet*. **22**, 64-71 (2009).

Totlani VM & Peterson DG. Epicatechin carbonyl-trapping reactions in aqueous maillard systems: Identification and structural elucidation. *J. Agric. Food Chem.* **54**, 7311-7318 (2006).

Vaidyanathan JB & Walle T. Glucuronidation and sulfation of the tea flavonoid (-)-epicatechin by the human and rat enzymes. *Drug Metab. Dispos*. **30(8)**, 897-903 (2002).

Vander Jagt DL & Hunsaker LA. Methylglyoxal metabolism and diabetic complications: roles of aldose reductase, glyoxalase-I, betaine aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenase. *Chem. Biol. Interact.* **143-144**, 341-351 (2003).

Vlassara H & Palace MR. Diabetes and advanced glycation endproducts. *J. Intern. Med.* **251**, 87-101 (2002).

Wijeratne SS, Abou-Zaid MM, & Shahidi F. Antioxidant polyphenols in almond and its coproducts. *J. Agric. Food Chem.* **54**, 312-318 (2006).

Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, & Prior RL. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.* **52**, 4026-4037 (2004).

Yamashiro J, Shiraishi S, Fuwa T, & Horie T. Dimerumic acid protected oxidative stress-induced cytotoxicity in isolated rat hepatocytes. *Cell. Biol. Toxicol*. **24(4)**, 283-290 (2008).

Yen GC & Duh PD. Scavenging Effect of Methanolic Extracts of Peanut Hulls on Free-Radical and Active-Oxygen Species. *J. Agric. Food Chem*. **42**, 629-632. 2008.

Yurttas HC, Schafer HW, & Warthesen JJ. Antioxidant activity of non-tocopherol hazelnut (*Corylus spp.*) phenolics. *J. Food Sci.* **65(2)**,276-280 (2000).

Zaveri NT. Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. *Life Sci*. **78**, 2073-2080 (2006).

Zhou JR & Erdman JW Jr. Phytic acid in health and disease. *Crit. Rev. Food Sci. Nutr.* **35(6)**, 495-508 (1995).

Zibaeenezhad MJ, Shamsnia SJ, & Khorasani M. Walnut consumption in hyperlipidemic patients. *Angiology*. **56(5)**, 581-583 (2005).