THE HEALTH CONSEQUENCES OF FRUCTOSE, ITS METABOLITE, DIHYDROXYACETONE AND THE HEPATOPROTECTIVE EFFECTS OF SELECTED NATURAL POLYPHENOLS IN RAT HEPATOCYTES

BY

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A thesis submitted in the conformity with the requirements

For the degree of Master of Science

Graduate Department of Pharmaceutical Sciences

University of Toronto

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ABSTRACT
THE HEALTH CONSEQUENCES OF FRUCTOSE, ITS METABOLITE, DIHYDROXYACETONE AND THE HEPATOPROTECTIVE EFFECTS OF SELECTED NATURAL POLYPHENOLS IN RAT HEPATOCYTES
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The introduction of high fructose corn syrup into the diet has been proposed to be the cause of many illnesses related to the metabolic syndrome. Fructose and its metabolites can be metabolized into cytotoxic reactive dicarbonyls that can cause damage to macromolecules leading to deleterious consequences. Dihydroxyacetone, a fructose metabolite, was studied in this thesis. Its ability to autoxidize and cause protein carbonylation under standard (pH 7.4, 37°C) and oxidative stress conditions (Fentons reagent) was investigated. Dihydroxyacetone was able to form significant amounts of dicarboxyls and protein carbonylation. Several selected natural polyphenols were chosen for an in vitro toxicological study involving rat hepatocytes. The chosen dietary polyphenols were rutin, gallic acid, methylgallate, ethylgallate, propylgallate and curcumin. In this thesis, the polyphenols were found to be able to significantly protect against the deleterious effects of glyoxal and methylglyoxal. In summary, these polyphenols could be candidates for future in vivo studies.
ACKNOWLEDGEMENTS
I would like to dedicate this thesis to my entire family. To my parents, I would like to thank them for encouraging my pursuit into the field of scientific research. I would also thank them for supporting me during my undergraduate years at University of Toronto.

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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-product</td>
</tr>
<tr>
<td>AGE-R1</td>
<td>P60</td>
</tr>
<tr>
<td>AGE-R2</td>
<td>80K-H phosphorprotein</td>
</tr>
<tr>
<td>AGE-R3</td>
<td>Galectin-3</td>
</tr>
<tr>
<td>ALDH2</td>
<td>Mitochondrial aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ALE</td>
<td>Advanced lipoxidation end-product</td>
</tr>
<tr>
<td>ApoB</td>
<td>Apoprotein B</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CCAC</td>
<td>Canadian Council on Animal Care</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>CEL</td>
<td>N-(carboxyethyl)lysine</td>
</tr>
<tr>
<td>CML</td>
<td>N-(carboxymethyl)lysine</td>
</tr>
<tr>
<td>COX-1</td>
<td>Cyclo-oxygenase I</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclo-oxygenase II</td>
</tr>
<tr>
<td>DHA</td>
<td>Dihydroxyacetone</td>
</tr>
<tr>
<td>DHA-P</td>
<td>Dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>DCF</td>
<td>Dichlorofluorescein</td>
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<tr>
<td>DCFD</td>
<td>Dichlorofluorescein diacetate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNPH</td>
<td>2,4-dinitrophenylhydrazine</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>Ferrous iron</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione sulfide</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>Glucose transporter type 4</td>
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<tr>
<td>GLUT-5</td>
<td>Glucose transporter type 5</td>
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<tr>
<td>GO</td>
<td>Glyoxal</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5-triphosphate</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>HEPES</td>
<td>Hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) hemisodium salt</td>
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<td>HFCS</td>
<td>High fructose corn syrup</td>
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IκB  Inhibitor of kappa B
IGN-1  Insulin-like growth factor 1
IL-1  Interleukin 1
IL-6  Interleukin 6
IL-1B  Interleukin 1B
iNOS  Inducible nitric oxide synthase
IRS-1  Insulin receptor substrate 1

JNK  c-Jun terminal kinase
JNK/AP1  c-Jun N-terminal kinase/Activator protein 1

LDL  Low-density lipoprotein
MGO  Methylglyoxal
MKK  Mitogen-activated protein kinase
MMP  Mitochondrial membrane potential
MOG  Mono-oxygenase
MSR ScR-II  Macrophage scavenger receptor type 2

NADPH  Nicotinamide adenine dinucleotide
NAFLD  Non-alcoholic fatty liver disease
NASH  Non-alcoholic steatohepatitis
NF-κB  Nuclear factor kappa B

O₂•  Superoxide anion
•OH  Hydroxyl radical

RAGE  Receptor of advanced glycation end-product
ROS  Reactive oxygen species

STAT-3  Signal transducer and activator of transcription 3
TCA  Trichloroacetic acid
TNF-α  tumor necrosis factor alpha
TPI  Triosephosphate isomerase

US  United States
VLDL  Very low density lipoproteins
CHAPTER 1: GENERAL INTRODUCTION

Fructose is rapidly becoming the main constituent of sweeteners in the Western diet. The increasing role of fructose in the Western diet has prompted a myriad of research into its metabolic fate and consequences to human health. Understanding the mechanisms by which fructose and its metabolites can lead to health complications and the role of oxidative stress in potentiating fructose toxicity is important in discovering novel strategies for combating and preventing fructose-induced toxicity. Natural polyphenols have recently gained interest because of their antioxidant properties. There are several classes of natural polyphenols found in a variety of foods consumed. Due to their antioxidant properties and abundance in foods, the prevention of fructose-related health complications by natural polyphenols was investigated.

1.01 The Western diet and fructose

The Western dietary pattern contains large amounts of red and processed meat, fried foods, refined grains, confectionery, high-fat dairy products, soft drinks and sweets (Maghsoudi and Azadbakht, 2012) and has been associated with the metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) and type II diabetes in adults (Esmailzadeh et al., 2007; Oddy et al., 2013; Qi et al., 2009). Major undesirable components of this Western diet include saturated fat, cholesterol, heme iron and more recently fructose (Qi et al., 2009). Under certain processes, these foods can generate advanced glycation and lipoxidation end products that are toxic to various cell types including pancreatic β cells (LeDoux et al., 1986). Although the mechanism(s) that cause an association between the Western dietary pattern and risk of metabolic syndrome is poorly understood, their association is also likely to be related to other constituents of healthy foods such as dietary fibre, vitamin E, folate and magnesium that are found in a variety of fruits,
vegetables and whole grains (Esmailzadeh et al., 2007). Reduced insulin demand may explain the beneficial effects of these nutrients (Esmailzadeh et al., 2007). High glycemic index foods have been shown to increase hepatic fat storage in animal studies (Scribner et al., 2007). In humans, high glycemic index foods have been associated with increased hepatic steatosis, particularly in insulin-resistant subjects (Valtuëña et al., 2006). An increased plasma glucose level leads to increased hepatic fat production via the de novo lipogenesis pathway (Le and Bortolotti, 2008). Indeed, obese subjects with a raised insulin level fed a Western style diet have been shown to have a higher rate of de novo lipogenesis (Schwarz et al., 2003). In contrast, the physical forms and high viscous fibre content of fruits and vegetables lead to slower digestion and absorption. Thus, these foods have a lower glycemic index, which is associated with a lower risk of insulin resistance (McKeown et al., 2004).

Fructose consumption has increased significantly in Western diets during the past few decades. Concurrently, obesity has risen over this time (the past 35 years) (Anderson, 2007). The food disappearance data, an indicator of food consumption trends, showed a 20% increase in caloric sweeteners (sugars) in the United States (US) from 1970 to 2000 (USDA, 2006). The introduction of high fructose corn syrup (HFCS) in beverages has been implicated (Bray et al., 2004). The introduction of HFCS was proposed to lead to obesity because fructose bypasses bodily regulatory systems and favours de novo lipogenesis (Anderson, 2007). Sucrose consumption rapidly declined from 80% of the total caloric sweeteners in 1970 to 40% in 1997 (Anderson, 2007), whereas HFCS increased from 0% in 1970 to 40% of total caloric sweeteners in 1997. However, changes in the availability of HFCS did not change from 1997 to 2004, during a time of continued population weight gain (Anderson, 2007). In addition, there was no
evidence that the ratio of fructose to glucose consumption changed over the past 4 decades as a result of HFCS. Nevertheless, few nutrients have received the amount of attention that fructose has received in the past 30 years (White, 2013).

HFCS was used as a substitute for sucrose in carbonated beverages, baked goods, canned fruits, jams and jellies and dairy products (Hanover and White, 1993). HFCS was sold in two forms: HFCS-55, representing 61.2% of all HFCS, containing 55% fructose, 41% glucose and 4% glucose polymers added to beverages and HFCS-42 containing 42% fructose, 53% glucose and 5% glucose polymers added to solid foods (Anderson, 2007). HFCS was produced from the catalysis of dextrose to glucose and various amounts of fructose by glucose isomerase (Bray et al., 2004). HFCS was inexpensive and it was profitable to use it to replace sucrose and simple sugars (Bray et al., 2004). In addition, HFCS has a similar composition, energy and sweetness to sucrose; it is easy to handle, is maintained in consistent supply, has superior functionality and saves on cost (White, 2013). HFCS is a major source of fructose and with the introduction of HFCS, estimated total fructose intake increased from 8.8% in 1977 to 11.5% in 1998 (Bray et al., 2004). Fructose has been hypothesized to be at the root of many American health problems. Evidence to support this include: 1) many significant diseases – obesity, diabetes, cardiovascular diseases, hypertension, cancer, NAFLD, metabolic syndrome – are related to fructose metabolism; 2) at typical human exposure levels and intake patterns, there is a cause-and-effect association between fructose metabolism and the above mentioned diseases (White, 2013). As such, the consumption of fructose has led to many health concerns. Overall, consumption of fructose and HFCS was closely related to overweightness and obesity (Figure 1.1) (Bray et al. 2004).
With the introduction of fructose to the Western diet, much research has been focused on understanding the metabolic and systemic fate of fructose in the human body.

**1.02 Fructose absorption and metabolism**

Diasaccharides like sucrose and maltose are absorbed by the intestine where they are cleaved by disaccharidases (Bray et al., 2004). A sodium-glucose co-transporter(s) causes the uptake of glucose. However, fructose is taken up further down the intestine by a non-sodium-dependent process in the duodenum and jejunum (Bray et al., 2004). Fructose then enters the portal circulation and is taken up by the liver (Bray et al., 2004).
Glucose enters the cells via glucose transporter type 4 (GLUT-4), which is insulin-dependent in most tissues (Bray et al., 2004). Insulin activates the insulin receptor leading to production of GLUT-4 (Bray et al., 2004).

In comparison with glucose uptake and metabolism, fructose is taken up by the insulin-independent transporter, glucose transporter type 5 (GLUT-5). GLUT-5 is absent from pancreatic β cells and the brain. Thus, while glucose provides satiety signals to the brain, fructose cannot (Bray et al., 2004). Additionally, insulin increases leptin secretion. Insulin can modify food intake by leptin secretion, which is regulated by insulin-dependent changes in glucose metabolism in adipose tissues (Mueller et al., 1998). Fructose does not stimulate insulin secretion because pancreatic β cells lack GLUT-5 (Bray et al., 2004). Therefore, the low insulin concentrations after fructose consumption leads to lower leptin concentration in comparison to post-glucose consumption (Bray et al., 2004). Leptin inhibits food uptake and lower leptin concentration leads to enhanced food intake (Bray et al., 2004).

The glucose metabolic pathway differs from the fructose pathway (Figure 1.2).
Figure 1.2. A simplified metabolic pathway for fructose and glucose. Glucose glycolysis is regulated by phosphofructokinase. Excess glucose is converted to glycogen. Fructose can bypass phosphofructokinase and undergo glycolysis. Also, fructose can further contribute to lipogenesis by providing glycerol-3-phosphate and acetyl-CoA (Adapted from Havel et al. 2005).
Glucose glycolysis is tightly regulated in three ways: 1) glucose-6-phosphate can be converted to glycogen; 2) glucose can be reformed from triose phosphates via gluconeogenesis and most importantly 3) the glucose glycolytic pathway is regulated by phosphofructokinase (Rutledge and Adeli, 2007). Phosphofructokinase is regulated by allosteric inhibition by citrate and adenosine triphosphate (ATP) (Figure 1.2) (Havel, 2005). In contrast, fructose is able to bypass the regulatory enzyme. This allows fructose carbons to enter glycolysis, glycogenesis, gluconeogenesis, lipogenesis, and fatty acid esterification. The carbons are eventually used for synthesis of glycerol and fatty acids, which are esterified to form triglycerides and subsequently very low density lipoproteins (VLDLs) (Figure 1.2) (Bray et al., 2004; Rutledge and Adeli, 2007). After consumption of a high fructose meal, for example consumption of HFCS-sweetened beverages, a significant amount of fructose carbons bypass phosphofructokinase and undergo glycolysis (Havel, 2005). Fructose is converted to fructose-1-phosphate catalyzed by fructokinase and ATP (Bray et al., 2004). Fructose-1-phosphate is then cleaved by aldolase B to form trioses such as glyceraldehyde, dihydroxyacetone-phosphate (DHA-P), and glyceraldehyde-3-phosphate (Rutledge and Adeli, 2007). However, some of the triose phosphates can undergo gluconeogenesis (Rutledge and Adeli, 2007). As the fructose glycolytic pathway becomes saturated, glycerol-3-phosphate is converted to the glycerol moiety, which is used for triglyceride synthesis (Havel, 2005). Alternatively, the triose phosphates can be further metabolized to pyruvate and to citrate and acetyl-CoA via pyruvate dehydrogenase in the mitochondria, where they are used for de novo lipogenesis and long-chain fatty acid synthesis (Havel, 2005). Unlike glucose which is regulated at the phosphofructokinase step, the majority of fructose carbons are used to produce acetyl-CoA (Havel, 2005).
After fructose is converted into glyceraldehyde and DHA-P, the trioses then undergo a series of reactions to form oxalate. This pathway involves a series of metabolic intermediates: glyoxylate, glycolate, glycoaldehyde, glyceraldehyde, hydroxypyruvate and glycerate (Figure 1.2) (Danpure and Rumsby, 2004). Glyoxal (GO) and methylglyoxal (MGO) are reactive dicarbonyls formed from the autoxidation of some of the fructose metabolites. The reactive dicarbonyls, GO and MGO, are detoxified by the glyoxalase system which is comprised of two enzymes. GO is converted to glycolate and MGO is converted to D-lactate using glutathione (GSH) as a cofactor (Thornalley, 1998). At first hemithioacetal is formed non-enzymatically from the α-oxoaldehydes with GSH (Abordo et al., 1999). Next, glyoxalase I catalyzes the formation of S-2-hydroxyacylglutathione derivatives from hemithioacetals (Abordo et al., 1999). Finally, glyoxalase II catalyzes the hydrolysis of S-2-hydroxyacylglutathione derivatives to form aldonates and GSH. The rate of formation of S-2-hydroxyacylglutathione derivatives is proportional to the cytosolic concentration of GSH. Under oxidative stress, the GSH level decreases causing glyoxalase I activity to also decrease. This leads to the accumulation of GO and MGO (Abordo et al., 1999). Due to the nature of the fructose glycolytic pathway, a high fructose diet, like in a Western diet, can lead to detrimental health consequences.

1.03 Health concerns

At elevated fructose concentrations, fructose promotes metabolic changes that are deleterious in rats and humans (Mayes, 1993). These include hyperlipidemia, hyperinsulinemia, hyperuricemia, lactacidemia and alterations in copper metabolism (Mayes, 1993). In animal models and human studies, fructose intake has been shown to be an important factor in the development of metabolic syndrome (Miller and Adeli, 2008). Rats fed with a fructose diet
developed hypertension and hyperinsulinemia (Hwang et al., 1987). Hamsters fed with a fructose diet developed insulin resistance and hypertriglyceridemia (Barros et al., 2007). In humans, a fructose diet led to increased postprandial plasma lipids, hepatic insulin resistance, increased lipid production and decreased lipid oxidation (Jeppesen et al., 1995; Wei et al., 2007; Chong et al., 2007).

Fructose and lipid metabolism are closely linked to each other (Miller and Adeli, 2008). Fructokinase, aldolase B and triokinase allow fructose to be converted to triose phosphates and bypass the phosphofructokinase regulatory step in glycolysis (Figure 1.2) (Mayes, 1993; Miller and Adeli, 2008). This allows fructose to enter glycolysis leading to the production of glycerol and fatty acids (Rutledge and Adeli, 2007). Triglyceride is formed from the esterification of these products (Mayes, 1993; Rutledge and Adeli, 2007). Triglyceride forms very-low density lipoprotein in the liver which is released into the bloodstream (Mayes, 1993). Upon hydrolysis, non-esterified fatty acids and monoacylglycerol are formed and taken up into adipose tissues where they are re-synthesized into triglyceride (Mayes, 1993). Hence, excessive fructose consumption leads to hyperlipidemia, hypertriglyceridemia and obesity. In normal glucose metabolism, triglyceride formation is inhibited by conversion of glucose to glycogen and reformation of glucose via gluconeogenesis which is tightly regulated by phosphofructokinase (Mayes, 1993).

Insulin resistance has been attributed to three fructose-related complications: 1) increased uptake of non-esterified fatty acids by liver; 2) induction of hepatic inflammation; 3) defects in the hepatic molecular signaling cascades (Rutledge and Adeli, 2007). Increased levels of
triglyceride and non-esterified fatty acids lead to impaired utilization of glucose in skeletal muscles (Mayes, 1993). Thus, decreasing glucose tolerance and increasing insulin resistance leads to hyperinsulinemia (Mayes, 1993).

Hepatic inflammation from chronic activation of the innate immune system contributes to the development of obesity and insulin resistance (Lazar, 2006). Increasing evidence has shown that macrophages play a role in the pathogenesis of insulin resistance and subsequent progression to fatty liver disease (Choi and Diehl, 2005). Macrophages infiltrate the adipose tissues and liver and produce cytokines, leading to inflammation (Choi and Diehl, 2005). Excess pro-inflammatory mediators, such as tumor necrosis factor – alpha (TNF-α) and interleukin-6 (IL-6), are found in patients with metabolic syndrome (Li and Diehl, 2003). c-Jun terminal kinases (JNKs) are serine/threonine kinases that promote the production of transcription factors such as c-Jun when activated by mitogen-activated protein kinase (MKK) 7 (Rutledge and Adeli, 2007). TNF-α activates JNK with high levels of non-esterified fatty acids (Hirosumi et al., 2002). JNK is also activated by fructose feeding and oxidative stress in rats (Wei and Pagliassotti, 2004). The activation of JNK leads to inhibitory phosphorylation of insulin receptor substrate-1 (IRS-1) at Ser-307, thereby interfering with the phosphorylation of tyrosine that is necessary for propagating the insulin signaling cascade (Rutledge and Adeli, 2007). Increased JNK activity was found in obese mice and in muscle samples of humans with insulin resistance (Hirosumi et al., 2002; Bandyopadhyay et al., 2005). Another mediator of fructose-induced insulin resistance is the nuclear factor kappa B (NF-κB). NF-κB enters the cell nucleus upon release from its inhibitor, IκB, inducing transcription of pro-inflammatory cytokines such as plasminogen activator inhibitor-1, TNF-α, IL-6 and interleukin-1B (IL-1B) (Cai et al., 2005). In addition,
fructose has been shown to activate signal transducer and activator of transcription-3 (STAT-3) and increase NκxB levels (Roglans et al., 2007). As described earlier, a high fructose diet is linked to fat accumulation and inflammation. These two factors have been linked to the development of NAFLD, which can result in non-alcoholic steatohepatitis (NASH), a more severe life-threatening form of NAFLD (Day and James, 1998). A two-hit hypothesis for the development of NASH has been proposed. First, the development of hepatic steatosis is followed by oxidative stress from inflammation or exposure to toxic xenobiotics (Day and James, 1998). Fructose can bypass the regulatory enzyme phosphofructokinase, leading to an increase in fructose-derived carbon and subsequent lipogenesis and steatosis (Rutledge and Adeli, 2007). The oxidative stress mechanism derives from reactive oxygen species (ROS)-induced oxidative stress. A high fructose diet can induce a hepatic response through the c-Jun N terminal Kinase/Activator Protein-1 (JNK/AP1) pathway, leading to inflammation and oxidative stress (Kelly et al., 2004). Also, under oxidative stress (simulated with a non-toxic concentration of H₂O₂), fructose hepatotoxicity was increased 125-fold (Lee et al., 2009). Fructose toxicity has been studied in in vivo experiments. When rats were infused with 0.55-2.2 mM fructose for an hour, liver transaminase enzymes, indicators of liver damage, were detected in the blood (Yu et al., 1974). In humans, 250mg/kg of injected fructose caused a decrease in hepatic ATP, increased inorganic phosphate and fructose phosphates (Oberhaensli et al., 1986) and increased plasma uric acid levels (Cirillo et al., 2006). Also, fructose consumption by human subjects with aldolase B deficiency led to possible liver and kidney toxicity (Gaby, 2005). The consumption of sucrose by male subjects for 18 days (25-30% of total calories) caused increased serum transaminases, indicating the presence of liver damage (Gaby, 2005).
Dietary fructose is associated with increased plasma uric acid levels (Miller and Adeli, 2008). A high plasmic uric acid level is associated with metabolic syndrome (Heinig and Johnson, 2006). Hyperuricemia derives from the utilization of ATP in phosphorylation of fructose and sequestering of inorganic phosphate into fructose-1-phosphate; thus, preventing the regeneration of ATP from adenosine diphosphate (ADP) (Mayes, 1993). This leads to the depletion of the total adenine nucleotide pool because enzymes of adenine nucleotide degradation are inhibited by ATP and inorganic phosphate. Thus, there is an increase in plasma uric acid levels. Hyperuricemia reduces the blood level of the vasodilator nitric oxide, leading to hypertension (Nakagawa et al., 2005). In addition, nitric oxide plays a role in hyperinsulinemia. Insulin increases the nitric oxide level to dilate blood vessels, enabling delivery of glucose to skeletal muscles (Heinig and Johnson, 2006). When nitric oxide secretion is impaired, more insulin is released to compensate (Heinig and Johnson, 2006). Additionally, a high fructose diet is linked to alterations in mineral metabolism. Fructose can complex with metal ions and modulate their absorption and bioavailability of minerals (O’Dell, 1993). In rats, fructose was found to decrease copper absorption and increase iron absorption (Johnson, 1986; Reiser et al., 1985).

Due to the metabolic potential of fructose to form toxic metabolites, it is necessary to understand its biochemical mechanisms involved to make available preventive or treatment options.

1.04 Maillard reaction and carbonyl stress

The Maillard reaction was discovered by the French biochemist Louis-Camille Maillard in 1912 (Henle et al., 1996). Maillard observed that reducing sugars can react with a wide array of proteins producing a brown colour (Gugliucci, 2000; Henle et al., 1996). The products of the reactions are low molecular weight heterocyclic flavor compounds or high molecular weight...
brown pigments called melanoidines (Ledl and Schleicher, 1990). More importantly, the Maillard reaction \textit{in vivo} produces advanced glycation end-products (AGEs) or advanced lipoxidation end-products (ALEs) (Figure 1.3) (Banach et al., 2009; Kalousová et al., 2004).
Figure 1.3. The non-enzymatic glycation of sugar. Non-enzymatic glycation can begin with any reducing sugar. The reducing sugar can react with an amine group on an amino acid (i.e. lysine or arginine) to form a reversible Schiff base. The Schiff base can undergo some partially reversible rearrangements to form an Amadori product. The Amadori product can undergo further modifications to form AGEs. Sugar, Schiff base and the Amadori product can form reactive dicarbonyls which can also form AGEs. Proteins can be modified by reducing sugars as well as reactive dicarbonyls (Adapted from O’Brien et al. 2005 & Busch et al. 2010).
Reducing sugars with carbonyl groups such as glucose or fructose and their metabolites react with free amino groups of proteins (Kalousová et al., 2004). Some of the amino acids targeted are arginine, lysine and cysteine (O’Brien et al., 2005). These reactions are dependent on glucose concentration in the blood and form a reversible Schiff base (Banach et al., 2009; Kalousová et al., 2004). The Schiff base then forms a partially reversible Amadori product such as fructosamine and glycated hemoglobin (Banach et al., 2009; Kalousová et al., 2004). After weeks or months, independent of sugar concentration, the Amadori products form AGEs via condensation, dehydration, fragmentation and cyclization (Banach et al., 2009; Kalousová et al., 2004). Sugars, Schiff bases and Amadori products can also undergo autoxidation to form reactive dicarbonyls such as GO and MGO to form AGEs (Kalousová et al., 2004). GO is formed from the metal or non-metal catalyzed autoxidation of Fenton’s catalyzed oxidation of glucose or fructose and their metabolites (O’Brien et al., 2005; Kalousová et al., 2004). MGO are formed from the triose phosphate glycolytic intermediates of glucose or fructose metabolism (Kalousová et al., 2004). These reactive dicarbonyls can also react with protein residues such as lysine and arginine leading to the formation of AGEs (O’Brien et al., 2005; Kalousová et al., 2004). These compounds can react with macromolecules leading to cytotoxicity (Kalousová et al., 2004). In diabetics, plasma GO and MGO levels are elevated and AGEs are found (Thornalley et al., 1999; O’Brien et al., 2005). The term carbonyl stress derives from circumstances where reactive carbonyl formation increased, or clearance or detoxification decreased, leading to reactive carbonyl overload (Baynes and Thorpe, 1999). Health complications could arise from the carbonyl stress and the formation of AGEs.
1.05 AGEs and health complications

AGEs are produced from the chemical modifications of proteins, lipids and nucleic acids by reducing sugars and reactive dicarboxyls as described above (Busch et al., 2010). AGEs are a class of heterogenous compounds that are characterized by brown colour, fluorescence and the tendency to polymerize (Gugliucci, 2000; Busch et al., 2010). Two main features of AGEs are that they have reduced solubility and are resistant to proteolysis (Busch et al., 2010). AGEs are formed naturally during aging, however, they are found at elevated levels in disease states such as diabetes mellitus, atherosclerosis, Alzheimer’s disease and renal failure (Vlassara and Palace, 2002; Busch et al., 2010). Proteins with long half-lives such as matrix and structural proteins are highly susceptible to modifications by AGEs. Traditionally, it was thought that AGEs form on long-lived extracellular proteins (Ahmed and Thornalley, 2007). However, it was recently found that proteins with short half-lives can be targeted as well (Busch et al., 2010). In particular, AGEs (hydroimidazolones), which are formed from α-oxoaldehydes, have relatively short half-lives (12-60 days) (Ahmed and Thornalley, 2007). Various types of AGEs exist, ranging from the early glycation adduct Nε-fructosyl-lysine to hydroimidazolones and monolysyl adducts (Ahmed and Thornalley, 2007). Physiologically, immunochemical evidence has indicated the presence of six AGE structures: AGEs-1 to 6 (Figure 1.4) (Takeuchi et al., 2001).
Figure 1.4. Alternative routes for formation of immunochemically distinct AGEs. AGEs arise from the degradation of the Amadori product (AGE-1), the glycolysis intermediate product glyceraldehyde (AGE-2), the Schiff base fragmentation product glycoaldehyde (AGE-3), the triose phosphate and the Amadori product fragmentation product methylglyoxal (AGE-4), the autoxidation product of glucose glyoxal (AGE-5), and the decomposition product of Amadori products and fructose-3-phosphate to 3-deoxyglucosone (AGE-6). CML, (N-(carboxymethyl)lysine; CEL, N-(carboxylethyl)lysine; p-NH2, free amino residue of protein; AR, aldose reductase; SDH, sorbitol dehydrogenase; 3-PK, fructose-3-phosphokinase; ALR, aldehyde reductase; GO, glyoxalase; MOG, mono-oxygenase (Takeuchi et al. 2001).
These AGEs were found in the serum of diabetic patients. Fructose and its metabolites, glyceraldehyde, glycoaldehyde, GO and MGO, were found to be linked to the production of AGEs-2 to 5 respectively (Takeuchi et al., 2001). In addition, fructose was found to form AGEs more readily than glucose (Ahmed and Thornalley, 2007). The body has several types of AGE scavenger receptors to regulate AGE levels leading to cell activation (Thornalley, 1998).

In diabetic patients, reactive carbonyls form at an increased rate due to the increased levels of sugars and triose metabolites (O’Brien et al., 2005). This leads to an increased AGE level leading to diabetic complications. AGE can accumulate in a variety of areas as a function of age and glycemia including the vascular wall, collagen, basement membranes, arterial wall, kidney mesangium and glomerulus (Gugliucci, 2000). AGE levels are significantly elevated in diabetic patients and in renally-impaired patients (Makita et al., 1991). Alterations of lipids and proteins by AGEs result in irregular molecule conformation, altered enzymatic activity and abnormal recognition and clearance by receptors (Ahmed and Thornalley, 2007). Alteration of lipids and lipoproteins leading to dyslipidemic changes are evident in diabetic patients (Vlassara and Palace, 2002). Lipids such as phosphatidylserine and phosphatidylethanolamine have amino groups that are susceptible to modification (Vlassara and Palace, 2002). The apoprotein B (ApoB) component of low-density lipoprotein (LDL) has abundant lysine and arginine sites for potential modification. AGE-ApoB levels are approximately 4 times higher in diabetic patients compared to a healthy individual (Bucala et al., 1993). In an animal study, transgenic mice expressing human LDL receptor were injected with AGE-LDL compounds. AGE-LDL showed delayed clearance compared to normal LDL (Bucala et al., 1994). The pathophysiological implication of this study is that hyperlipoproteinemia can contribute to atherosclerosis by
reducing LDL clearance and accumulation of AGE-LDL via AGE-receptor interactions at the vessel wall (Bucala et al., 1994). The arterial wall can trap LDL in the intima (Gugliucci, 2000). In this way, LDL is prone to oxidation. Modification of LDL by AGEs has been shown to increase LDL susceptibility to oxidation and enhance the propensity for LDL to bind to vessel walls (Vlassara, 1995). Oxidized LDL can be taken up by monocytes/macrophages, producing an immune-cell mediated pro-inflammatory response by cytokine release (Gugliucci, 2000). At the same time, endothelial cells can be activated by oxidized LDL, mediating the deposition of atheroma (Witztum and Steinberg, 1991). Glycated and oxidized LDL cause cholesteryl ester accumulation in human macrophages and promote platelet and endothelial cell dysfunction (Vlassara, 1995). Consistent with this, increased levels of LDL greatly predispose diabetic patients to atherosclerosis (Vlassara and Palace, 2002). Finally, AGEs produce a dose-dependent decrease in nitric oxide (NO), a vasodilator, levels (Gugliucci, 2000).

The rate of formation of AGE does not follow first-order kinetics. Thus, a modest increase in hyperglycemia in diabetics can result in significant AGE accumulation in long-lived macromolecules (e.g. proteins in the ocular lens) (Gugliucci, 2000; Vlassara and Palace, 2002). This accounts for opacification and cataract formation in aging and diabetes (Monnier et al., 1979). Proteins in the extracellular matrix and vascular basement membranes are some of the longest lived in the body and are highly susceptible to modification by AGEs (Charonis and Tsilbary, 1992). AGEs form covalent, intermolecular bonds in collagen, causing luminal narrowing, a major feature of diabetic blood vessels (Gugliucci, 2000; Tanaka et al., 1988). Additionally, plasma membranes such as albumin, lipoprotein, and immunoglobulin may get trapped in the basement membrane by crosslinking to AGEs on collagen (Brownlee et al., 1985).
Laminin (a structural protein) modified by AGEs caused a reduction in polymer self-assembly and decreased binding of scaffolding proteins such as type IV collagen and heparan sulfate proteoglycan (Makino et al., 1995). Heparan sulfate proteoglycan is a negatively charged glycoprotein that impairs leakage of plasma proteins (Striker et al., 1991). The diabetic-induced loss of heparan sulfate proteoglycan can result in proteinuria and overproduction of membrane proteins (Makino et al., 1995). Proteinuria, mesangial expansion and focal sclerosis are the main features of diabetic glomerulopathy (Gugliucci, 2000).

The AGEs are removed from the body and elicit their physiological effects via numerous receptors and binding proteins (Gugliucci, 2000; Vlassara and Palace, 2002). The AGE receptors include receptor of advanced glycation end-product (RAGE), AGE-R1 (OST-48, p60), AGE-R2 (80K-H phosphoprotein), AGE-R3 (galectin-3), MSR ScR-II (macrophage scavenger receptor type 2) and CD36 (cluster of differentiation 36). Amongst these AGE receptors, RAGE is the most studied. RAGE is a multi-ligand receptor and is a member of an immunoglobulin superfamily. RAGE expression has been found in endothelial cells, vascular smooth muscle cells, mononuclear cells, neural tissues, lungs and skeletal muscle (Brett et al., 1993). Although RAGE is not efficient in AGE endocytosis and turnover, RAGE plays a role in intracellular signal transduction or as a pro-inflammatory peptide (Vlassara and Palace, 2002). In animal models, truncated RAGE causes physiological processes such as endothelial leakage, atherosclerosis and inflammatory bowel disease (Schmidt et al., 1999).

Monocytes and macrophages possess RAGE (Gugliucci, 2000; Busch et al., 2010). RAGE stimulates the secretion of pro-inflammatory cytokines such as interleukin-1 (IL-1), insulin-like
growth factor-1 (IGF-I), TNF-α, and granulocyte/macrophage colony stimulating factor, leading to the production of type IV collagen causing proliferation of arterial smooth muscle cells and macrophages (Bierhaus et al., 1998). Additionally, AGEs that bind to the endothelium can lead to the activation of NF-κB and subsequent transcription of “response-to-injury” genes. Thrombic forms at sites of AGE accumulation due to these AGE-induced changes involving thrombomodulin modification and tissue factor production (Wautier et al., 1996). Also AGE-RAGE interaction induces RAGE upregulation because the promoter region contains NF-κB binding sites (Li and Schmidt, 1997). TNF-α may also cause RAGE expression through NF-κB (Busch et al., 2010). In summary, the colocalizations of RAGE and AGE at microvascular injury sites suggest their role in the pathogenesis of diabetic vascular lesions (Bierhaus et al., 1997). During diabetic nephropathy, RAGE is upregulated on podocytes in human kidney (Busch et al., 2010). Renal endothelial cells also express RAGE and may play a role in renal vascular injury (Yan et al., 2009). Activation of RAGE stimulates the secretion of platelet-derived growth factor (PDGF); mediating mesangial expansion (Skolnik et al., 1991).

Due to various deleterious effects of AGEs, compounds that can prevent or reduce the formation of AGEs could improve the clinical outcomes of various diseases. Theoretically, there are three methods to improve the outcomes of AGE-related diseases – prevent AGE formation, degrade AGEs or block the activation of RAGE (Busch et al., 2010). MGO and GO are viewed as precursors to the formation of AGEs. Therefore, therapeutic agents aimed at preventing their formation or scavenging them may prevent the development of AGE-related complications in diabetes (Aldini et al., 2013). Since reactive carbonyl species are electrophilic compounds, carbonyl scavengers tend to be nucleophilic (Aldini et al., 2013). Aminoguanidine is a
nucleophilic carbonyl scavenger. It has been shown to prevent AGE formation in the retina or glomeruli and improve nerve conduction speed in diabetic rats (Huijberts et al., 1994).

Understanding the formation and metabolism of AGEs and their precursors is beneficial and could facilitate the development of other therapeutic agents.

Carbonyl stress can cause the rapid formation of AGEs which can lead to oxidative stress through gene transcription pathways. Oxidative stress can induce further reactive dicarbonyl formation. In disease states such as hyperglycemia, ROS levels are elevated and autoxidation of sugars, Schiff base and Amadori products occurs, giving rise to reactive dicarbonyls, which contribute to AGE formation. These reactions can be enhanced and catalyzed by metal ions such as iron and copper (Bierhaus et al., 1998). Thus, understanding the interaction of carbonyls and oxidative stress could lead to the discovery of novel therapeutic strategies against the metabolic syndromes.

1.06 Oxidative Stress

Oxidative stress arises when there is an imbalance between levels of reactive oxygen species and levels of antioxidant systems. Most cells can tolerate a mild degree of oxidative stress with antioxidant defense and repair systems. However, when there is an over production or distribution of ROS, macromolecules such as lipids, proteins and deoxyribonucleic acid (DNA) will be damaged. ROS-induced damage can modulate gene expression, cell adhesion, cell cycle metabolism and cell death (Ha et al., 2010).
The three ROS of toxicological importance are superoxide anion (O$_2^•$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (•OH) (Figure 1.5).

\[ a) \; O_2 + e^- \longrightarrow O_2^• \]

\[ b) \; \text{NADPH} + 2 \; O_2 \quad \overset{\text{NADPH Oxidase}}{\longrightarrow} \quad \text{NADP}^+ + 2 \; O_2^- + H^+ \]

\[ c) \; 2 \; O_2^- + 2 \; H^+ \quad \overset{\text{Superoxide Dismutase}}{\longrightarrow} \quad O_2 + H_2O_2 \]

\[ d) \; \begin{array}{c}
Fe^{3+} + O_2^- \\
Fe^{2+} + H_2O_2 \\
O_2^- + H_2O_2
\end{array} \quad \overset{\text{Haber-Weiss}}{\longrightarrow} \quad \begin{array}{c}
Fe^{2+} + O_2 \\
Fe^{3+} + OH^- + •OH
\end{array} \quad \overset{\text{Fenton reaction}}{\longrightarrow} \quad \begin{array}{c}
O_2 + OH^- + •OH
\end{array} \quad \overset{\text{Net reaction}}{\longrightarrow} \quad \begin{array}{c}
\end{array} \]

Figure 1.5. The formation of the three major endogenous ROS. a) Superoxide anion can form from the addition of an electron to oxygen. b) Production of O$_2^•$ from the NADPH oxidase in polymorphonuclear leukocytes. c) O$_2^•$ can be converted to H$_2$O$_2$ through superoxide dismutase. d) •OH can be formed through a series of reactions – the Haber Weiss reaction followed by the Fenton reaction. NADP$^+$/NADPH, nicotinamide adenine dinucleotide (Adapted from Birben et al. 2012).

A O$_2^•$ is formed from the addition of one electron to oxygen (Figure 1.5a) (Miller et al., 1990). This reaction can be mediated by NADPH oxidase or xanthine oxidase or by the mitochondrial electron transport chain (Figure 1.5b) (Birben et al., 2012). In the mitochondria, reduction of oxygen to water occurs and 1-3% of all electrons leak and produce O$_2^•$ (Birben et al., 2012; Rosca et al., 2005). O$_2^•$ are detoxified by the enzyme, superoxide dismutase, which catalyzes the
formation of H$_2$O$_2$ from O$_2$• (Figure 1.5c) (Ha et al., 2010; Birben et al., 2012). In a Haber-Weiss reaction followed by a Fenton reaction, H$_2$O$_2$ can be broken down to •OH in the presence of transition metals iron or copper (Figure 1.5d) (Fenton, 1984). Additionally, superoxide can react with H$_2$O$_2$ to form hydroxyl radicals (Haber and Weiss, 1934).

ROS can participate in essential cellular functions such as cell signaling, cell migration, gene expression and cell growth (Martindale and Holbrook, 2002). However, ROS have also been linked to aging and pathologies including cancer and neurodegeneration (Halliwell, 2009). As described in earlier sections, fructose more readily forms reactive carbonyls than glucose (Suárez et al., 1989). As a result, fructose can initiate the start of an inflammatory cascade through the interaction of AGE and RAGE (Abordo et al., 1999). The NK-κB pathway can be stimulated by cytokines such as TNF-α and ILs via pro-inflammatory receptors (Czaja, 2007; Martindale and Holbrook, 2002; Morgan and Liu, 2011). NF-κB activation leads to the expression of various genes responsible for cell growth, development, immune function, inflammation and apoptosis (Martindale and Holbrook, 2002; Morgan and Liu, 2011). ROS can also activate the NF-κB pathway, furthering the production of cytokines (Martindale and Holbrook, 2002; Morgan and Liu, 2011). Additionally, NF-κB can increase the expression of oxidases, increasing the levels of ROS (Morgan and Liu, 2011). Mitochondrial respiratory complexes I, III and IV are the main targets of hyperglycemia-induced injury (Rosca et al., 2005). Under chronic hyperglycemia, as in diabetes patients, MGO can glycate and alter complex III, contributing to O$_2$• formation (Rosca et al., 2005). Polymorphonuclear leukocytes, monocytes and macrophages possess peroxisomal NADPH oxidase which play a role in bactericidal activity upon phagocytosis (Birben et al., 2012). Inducible nitric oxide synthase (iNOS) can generate nitric oxide which can
react with superoxide to form a reactive nitrogen species, peroxynitrite (Morgan and Liu, 2011). Peroxynitrite can then react with carbon dioxide to form nitrosoperoxy carbonate which can be broken down into nitrogen dioxide radicals and carbonate. Peroxynitrite is a highly reactive nitrogen species and can cause various cellular damages (Morgan and Liu, 2011). The •OH is the most reactive ROS and is capable of damaging macromolecules (Birben et al., 2012). In particular, it can cause lipid peroxidation by extracting an electron from polyunsaturated fatty acids (Birben et al., 2012). Various peroxisomal oxidases use various substrates such as amino acids, uric acid, xanthine and fatty acids to detoxify ROS (Angermüller et al., 2009).

Detoxifying enzymes including superoxide dismutase (mentioned above), catalase and GSH peroxidase inactivate H₂O₂ (Ha et al., 2010). Catalase is found in peroxisomes and catalyzes the formation of water and oxygen from H₂O₂ (Figure 1.6a). Additionally, it may also react with H₂O₂ and hydrogen donors, producing a water molecule and oxidation of the reduced donor (Ha et al., 2010).
Figure 1.6. The detoxification of $\text{H}_2\text{O}_2$. a) $\text{H}_2\text{O}_2$ is detoxified by the peroxisomal enzyme catalase. b) $\text{H}_2\text{O}_2$ is detoxified by the GSH dependent enzyme GSH peroxidase. Cofactor GSH is rejuvenated by the enzymes GSH reductase followed by glucose-6-phosphate dehydrogenase. NADP$^+$/NADPH, nicotinamide adenine dinucleotide phosphate (Adapted from Birben et al. 2012).

Under oxidative stress, dicarbonyl and ROS detoxifying systems can be interrupted due to depletion of cofactors such as GSH used by detoxifying enzymes (Morgan and Liu, 2011; Baynes and Thorpe, 1999). GSH is an antioxidant coenzyme used by GSH peroxidases to reduce peroxides or $\text{O}_2\cdot$ and form glutathione disulfide (GSSG) (Figure 1.6b) (Baynes and Thorpe, 1999). GSH is also used in the glyoxalase pathway to detoxify reactive dicarbonyls where dicarbonyls are rearranged into hydroxyacids (Yang et al., 2011b). GSH is also used in
the NADPH-dependent reduction of MGO by aldose reductase (Jones et al., 2000). Glyoxalase I converts MGO to lactate, while GSH forms a hemithioacetal intermediate carbonyl trap (Baynes and Thorpe, 1999). GSH is released from the conjugate as GSH, not GSSG (Baynes and Thorpe, 1999). In diabetes patients, MGO is elevated in the blood due to the decreased levels of GSH for the glyoxalase system (Baynes and Thorpe, 1999). The lack of GSH can be attributed to high levels of ROS in diabetic patients. High levels of ROS can disrupt cellular functions due to destruction of macromolecules and catalyze the production of various endogenous toxins (Morgan and Liu, 2011). To combat the toxicity of ROS, various agents are available to prevent the formation of ROS or scavenge ROS before they can induce toxicity. Natural polyphenols are a large group of natural antioxidants that scavenges ROS.

1.07 Polyphenols
Polyphenols are a group of secondary plant metabolites with at least one aromatic ring structure with one or more hydroxyl groups (van Duynhoven et al., 2011). Polyphenols are found in foods we eat daily such as fruits, tea, coffee, wine, fruit juices, chocolate, vegetables, cereals and legume seeds (Scalbert et al., 2002). Polyphenols have been extensively studied for their therapeutic effects. These include lowering risks of cancer, cardiovascular diseases, hypertension, neurodegenerative diseases and stroke (Wolfe et al., 2003). Polyphenols have been shown to function in the modulation of vascular and platelet function, lowering blood pressure and improving lipid profile (Hodgeson and Kroft, 2006). All polyphenols function as reducing agents (Scalbert et al., 2002; Scalbert and Williamson, 2000). They scavenge ROS, protect against inflammation, function as endothelial protectors, regenerate antioxidants such as vitamin E and protect against oxidative damage (van Duynhoven et al., 2011; Scalbert et al.,
2002). There are various groupings of polyphenols and they are classified by the structure of the carbon skeleton. These classes include flavonoids, phenolic acids, stilbenes and lignans (Table 1.1). The structure of the polyphenol affects its bioavailability, redox potential, antioxidant activity, interaction with cellular receptors and enzymes (Scalbert and Williamson, 2000). Additionally, the greater number of hydroxyl groups is associated with greater antioxidant capability (Scalbert et al., 2002).

Table 1.1 Classes of natural polyphenols and examples of each classes (Adapted from Scalbert et al. 2002 & Scalbert & Williamson 2000).

<table>
<thead>
<tr>
<th>Class</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flavonoids</strong></td>
<td></td>
</tr>
<tr>
<td>Flavonols</td>
<td>Rutin, quercetin, kaempferol, myricetin</td>
</tr>
<tr>
<td>Flavones</td>
<td>Luteolin, apigenin</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Daidzein, genistein</td>
</tr>
<tr>
<td>Flavanones</td>
<td>Hesperetin, naringenin</td>
</tr>
<tr>
<td>Flavanol</td>
<td></td>
</tr>
<tr>
<td>Catechins</td>
<td>Catechin, epicatechin, gallocatechin, epigallocatechin gallate</td>
</tr>
<tr>
<td>Proanthocyanidins</td>
<td>Procyanidins, prodelphinidins</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>Cyanidin, delphinidin</td>
</tr>
<tr>
<td><strong>Phenolic acids</strong></td>
<td></td>
</tr>
<tr>
<td>Cinnamic acids</td>
<td>Caffeic acid, ferulic acid, chlorogenic acid</td>
</tr>
<tr>
<td>Benzoic acids</td>
<td>Gallic acid</td>
</tr>
<tr>
<td>Ellagitannins</td>
<td>Casuarictin, sanguin H6,</td>
</tr>
<tr>
<td>Stilbenes</td>
<td>Resveratrol</td>
</tr>
<tr>
<td>Lignans</td>
<td>Enterodiol</td>
</tr>
<tr>
<td>Curcuminoid</td>
<td>Curcumin</td>
</tr>
</tbody>
</table>

Flavonoids are the most abundant polyphenol in our diet (Albarracin et al., 2012; Scalbert and Williamson, 2000). Flavonoids are further classified by the degree of oxidation of the oxygen heterocycle: flavonols, flavones, isoflavones, flavonones, anthocyanins, and proanthocyanidins. Tea polyphenols are part of the flavonoid group – they include rutin, quercetin, catechin and its derivatives (Albarracin et al., 2012). Tea polyphenols are strong scavengers of hydroxyl...
radicals, nitric oxide radicals and lipid oxidation (Panickar et al., 2009). The strong scavenging capability of catechin and its derivatives are related to the gallate moiety and hydroxyl group (Albarracin et al., 2012). Quercetin, the main flavonol, is abundant in onions (0.3mg/g) and tea (10-25mg/L) (Hertog et al. 1992; Hertog et al. 1993). Soy is a thoroughly studied isoflavone because soy contains 1mg genistein and daidzein/g dry bean (Reinli and Block 1996). These isoflavones have estrogenic properties that prevent breast cancer and osteoporosis (Adlercreutz and Mazur 1997). A major source of flavanones is citrus fruits. Oranges contain 125-250 mg/L juice of hesperidin (Rousseff et al. 1987). Flavones are less common. They are found in sweet red pepper as luteolin and celery as apigenin (Hertog et al. 1992). Proanthocyanidins are polymeric flavanols, which account for the astringent property of foods (Scalbert and Williamson, 2000). Proanthocyanidins are found in plants as mixtures of polymers and are usually present in association with catechin (Scalbert and Williamson, 2000). Some common sources of proanthocyanidins include apple, pear, grape, red wine, tea and chocolate (Santos-Buelga and Scalbert 2000). Anthocyanins are the red pigments of various fruits including cherries, plums, strawberries, raspberries and blackberries. Their levels vary from 0.15 – 4.5 mg/g (Scalbert and Williamson, 2000). Red wine is a major source of anthocyanins (26mg/L) (Frankel et al. 1995).

The next class of polyphenols, phenolic acids, is abundant in foods (Table 1.1). Phenolic acids are usually esterified to polyols such as glucose (Scalbert and Williamson, 2000). The most common phenolic acids are caffeic acid and ferulic acid, which are found in dietary fibre esterified to hemicelluloses (Kroon et al. 1997). Other phenolic acids include gallic acid in gallotannins and derivatives of galloyl residues in ellogallotannins (found in berries and wine)
Stilbenes are not widespread polyphenols. Resveratrol, found at a very low concentration in wine (0.3-2mg/L red wine), is part of the stilbene family (Frankel et al. 1995). Lignans are found in considerable amounts in flaxseed and flaxseed oil (Axelson et al. 1982). Lignan precursors have not been identified; however, the precursors are metabolized by human and animal gut microflora to lignans. Lignans are phytoestrogens with agonistic and antagonistic properties (Scalbert and Williamson, 2000). Curcumin is abundant in turmeric from the plant Curcuma longa Linn. It is a food additive and a traditional herbal remedy. It acts as an anti-inflammatory and antioxidant. Additionally, it has been shown to have anti-amyloidogenic effects (Hirohata et al., 2007). It binds to amyloids directly and inhibits their aggregation and the formation of fibril and oligomers that are common in Alzheimer’s disease and Parkinson disease patients (Yang et al., 2005). In recent times, curcumin has also been the focus of anti-cancer research.

Various cell models have demonstrated the antioxidant capacity of polyphenols. However, studies of bioavailability in humans and animals have led to doubt as to whether polyphenols have an antioxidant effect in vivo. However, indirect evidence of absorption of polyphenols comes from the observation of increased plasma antioxidant capacity after consumption of polyphenol-rich foods such as tea, red wine, or apple juice (Scalbert and Williamson, 2000). Direct evidence comes from measuring pure polyphenol contents in plasma or urine. Polyphenols are usually found in glycosylated form linked with glucose, rhamnose, galactose, xylose and glucuronic acid (Gee et al. 1998). The sugar group can also be further substituted. Linkage to sugar groups and substitution influences the chemical, physical and biological activity of the polyphenol. The human body requires enzymatic de-glycosylation (non-enzymatic de-
glycosylation does not occur) in order for the polyphenol to be absorbed (Gee et al. 1998). Polyphenols are de-glycosylated by β-glucosidases or by microflora α-rhamnosidases (Scalbert and Williamson, 2000). Depending on the glycosylation pattern and polymerization degree, polyphenols may be absorbed at different rates and extents and different metabolites are formed (van Duynhoven et al., 2011; Scalbert and Williamson, 2000). Thus, a significant amount of polyphenols may persist in the colon and be exposed to the large metabolic potential of the gut microbial community.

Polyphenol absorption is verified by the increased antioxidant capacity of the plasma following consumption (Fuhrman et al., 1995). Polyphenols and their metabolites are also recovered in the urine following consumption (Figure 1.7).
Figure 1.7. A simplified pathway of polyphenol absorption, metabolism and excretion. Dietary polyphenols are absorbed through the small intestine and the colon. The dietary polyphenol and its metabolites then enter the liver and is distributed into the bloodstream with antioxidant effects. The polyphenols are excreted as feces or via the kidney or into the bile (Adapted from Scalbert and Williamson 2000).

Some polyphenols have low recovery because they are stable in the gastric or intestinal juices or they are excreted into the bile (Gee et al., 1998). Polyphenols are conjugated to form O-glucuronides, sulfate esters or O-methyl ether before being absorbed (Scalbert et al., 2002). The first conjugation occurs at the gut barrier. This was demonstrated in rats where quercetin glucuronides were formed in the gut mucosa upon quercetin perfusion (Crespy et al., 1999).
Methylation occurs in the liver. For example, catechin is methylated in the liver (Scalbert et al., 2002). Conjugation facilitates the excretion of polyphenols. Glucuronidation reduces the antioxidant capacity of polyphenols while methylation affects the biological properties of polyphenols (Scalbert et al., 2002). Conjugates can also have biological activity at the cellular level upon de-conjugation (Scalbert et al., 2002). In the colon, there are approximately $10^{12}$ microorganisms per gram of gut content (van Duynhoven et al., 2011). Deglycosylation, dehydroxylation, demethylation and catabolism may occur in the colon (Cermak et al., 2006). De-conjugated polyphenols are further metabolized to low molecular weight aromatic acids such as phenylvaleric acid, phenylpropionic acid, phenylacetic acid and benzoic acid (Scalbert et al., 2002).

Polyphenols have variable absorption rates. Quercetin and rutin has a particularly low absorption rate (0.3-1.4% weight ingested) (Scalbert and Williamson, 2000). However, the absorption rate is much higher for catechins in green tea, isoflavones in soy, flavonones in citrus fruits and anthocyanidins in wine (3-26%) (Fuhr and Kummert 1995). A large percentage of polyphenols ingested are excreted into the urine (75-99%) (Scalbert and Williamson, 2000). The rest of the polyphenols are excreted into the bile or are metabolized by the gut microflora (Scalbert and Williamson, 2000). The gut lumen has a higher concentration of polyphenols than the plasma. The plasma concentrations rarely exceed 1 µM after ingesting 10-100 mg of any single polyphenol (Scalbert and Williamson, 2000). Some effects of the polyphenols are their protection of the gut mucosa against oxidative damage (Scalbert et al., 2002). For example, wine and tea polyphenols ingested by rats lower the oxidative stress on caecal mucosal cells (Giovannelli et al., 2000). Polyphenols can also interact with other nutrients in the gut lumen.
For example, polyphenols may form stable complexes with non-heme irons, which limit its absorption (Hurrell et al., 1999).

As reviewed above, polyphenols are antioxidants with the potential to become therapeutic agents for various diseases. Polyphenols can combat the effects of oxidative stress, which potentiates carbonyl stress. Through a series of reactions with sugars, reactive dicarboxyls can form AGEs. With many health complications related to elevated levels of AGEs the targeting of, or prevention of, the formation of AGEs is important. Sugars, such as glucose and fructose, can produce AGEs through undesirable routes of their glycolytic pathways. Fructose is rapidly becoming the main constituent of sweeteners in the Western diet and its detrimental consequences are becoming apparent. Hence, the prevention of fructose toxicity warrants further investigation.

1.08 Scope of thesis

Increased consumption of dietary fructose is linked to the development of metabolic syndrome which is a collection of disorders including dyslipidemia, insulin resistance and central adiposity (Dekker et al., 2010). Fructose and its metabolites, glycolaldehyde, glyceraldehyde, hydroxypyruvate and dehydroxyacetone, can be metabolized to form reactive dicarboxyls (Havel, 2005). Reducing sugars, such as fructose and glucose, and reactive dicarboxyls can form reversible Schiff bases which can form partially reversible Amadori products (Busch et al., 2010). Finally, through a series of rearrangements, dehydration, defragmentation, Amadori products can form stable AGEs (Busch et al., 2010). Fructose, reactive dicarboxyls and AGEs have been shown to increase oxidative stress. Fructose can form reactive dicarboxyls
enzymatically (Havel, 2005). Under oxidative stress conditions, fructose hepatotoxicity and the ability to form reactive dicarbonyls was potentiated (Lee et al., 2009). However, the ability of fructose to form significant amount of reactive dicarbonyls non-enzymatically in comparison with fructose metabolites is not known (Havel, 2005). It has been widely accepted that oxidative stress contributes to the development and progression of diabetes and its complications such as macro- and microangiopathy, neuropathy, nephropathy (Coriello, 2000; Maritim et al., 2003). An increase in oxidative stress reduces the detoxifying potential of the body. Oxidative stress can also increase the production of reactive dicarbonyls from reducing sugars (Lee et al., 2009; Maritim et al., 2003). Due to the detrimental effects of AGEs, preventing AGE formation is important. Some of the prevention techniques involve scavenging dicarbonyls, degrading AGEs and blocking the activation of RAGE that can trigger an inflammatory cascade (Aldini et al., 2013).

Natural polyphenols are a group of complex phytochemicals. Natural polyphenols are secondary plant metabolites derived from the shikimic pathway, a metabolic route used by plants for the biosynthesis of aromatic residues, and share at least one aromatic structure (van Duynhoven et al., 2011). Natural polyphenols are known antioxidants and have been the focus of research as potential therapeutic agent for various types of diseases (Albarracin et al., 2012; Scalbert and Williamson, 2000). Most studies use the native form of polyphenols. However, polyphenols have been shown to undergo various transformations before entering the bloodstream for systemic therapeutic effects (Scalbert and Williamson, 2000). As a result, the therapeutic effects of polyphenol metabolites are poorly understood.
1.09 Hypothesis

Background to Hypothesis 1 (Chapter 3):

Fructose consumption has increased significantly due to the introduction of HFCS. Thus, it is important to understand its metabolic fate and health sequences in the human body.

Dihydroxyacetone is a fructose metabolite upstream of the reactive dicarbonyls. Both fructose and dihydroxyacetone can form reactive dicarbonyls that may contribute to protein carbonylation.

Hypothesis 1: Dihydroxyacetone autoxidizes to form reactive dicarbonyls which contribute to protein carbonylation. In comparison with fructose, dihydroxyacetone can autoxidize and cause protein carbonylation to a much greater extent.

Background to Hypothesis 2 (Chapter 3):

Natural polyphenols are found in a variety of consumer foods. A number of natural polyphenols have been examined for their therapeutic properties against various diseases. GO and MGO causes oxidative stress by glycating essential proteins in the mitochondria and by initiating lipid peroxidation. Natural polyphenols may act as an antioxidant by relieving the oxidative stress caused by GO and MGO.

Hypothesis 2: Natural polyphenols are hepatoprotective against reactive dicarbonyls, GO and MGO, and prevent cytotoxicity, reducing ROS levels and maintaining normal MMP status.
Fructose metabolism mainly takes place in the liver. Hence, rat hepatocytes were chosen for the investigation. Chapter 3 described the metabolic potential of fructose and dihydroxyacetone to form reactive dicarbonyls and cause protein carbonylation. Chapter 3 also described the hepatoprotective effect of the natural polyphenols against GO and MGO cytotoxic effects.

1.10 Thesis Organization

This thesis has been organized in the manner of a traditional thesis with an Introduction (this Chapter 1) and Materials and Methods (Chapter 2), the Results and Discussion section (Chapter 3 of this thesis) is a paper with the author of this thesis as first author. It was published in the Journal “Chemico-Biological Interactions” (H. Lip, K. Yang, S.L. MacAllister, P.J. O’Brien, “Glyoxal and methylglyoxal: Autoxidation from dihydroxyacetone and polyphenol cytoprotective antioxidant mechanisms”, 202(2013) 267-274) (Lip et al. 2013). Chapter 3 is this publication verbatim with the Materials and Methods section removed. Chapter 4 is a Final Summary of the thesis and a Proposal for Future Work which includes preliminary results by the thesis author. Appendix A is the results of preliminary future works. Appendix B is the permission for reprint of article “Glyoxal and methylglyoxal: Autoxidation from dihydroxyacetone and polyphenol cytoprotective antioxidant mechanisms”.

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2.1 Chemicals
GO [40%, weight/volume(w/v)], MGO (40% w/v), 1-bromoheptane, gallic acid, methyl gallate, ethyl gallate, propyl gallate, rutin hydrate, curcumin, trans-ferulic acid, 4-hydroxy-3-methoxycinnamaldehyde, isoeugenol, quercetin, 3,4-dihydroxyphenylacetic acid, homovanillic acid, 4-methylcatechol, fructose, 1,3-dihydroxyacetone dimer, 2’,7’-dichlorofluorescein diacetate (DCFD), rhodamine 123, dimethylsulfoxide (DMSO), sodium phosphate dibasic, potassium phosphate monobasic, potassium chloride, calcium chloride, magnesium sulfate, sodium bicarbonate, N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) hemisodium salt, ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid, sodium phosphate monobasic, ferrous sulfate, ethylenediaminetetraacetic acid (EDTA), H₂O₂ (30%, w/v), anhydrous sodium borate, Girard’s T reagent, bovine serum albumin, 2,4-dinitrophenylhydrazine (DNPH) and trichloroacetic acid (TCA) were purchased from Sigma–Aldrich Corp. (Oakville, ON, CAN). Type II Collagenase was purchased from Worthington (Lakewood, NJ, USA). Stock solutions of chemicals were made up in H₂O or DMSO.

2.2 Animal treatment and hepatocyte preparation
Male Sprague-Dawley rats weighing 275 – 300 g (Charles River Laboratories, Senneville, QC, CAN) were housed in ventilated plastic cages with PWI8 – 16 hardwood bedding with 12 air changes per hour and 12 h of light (on at 08:00). All animal care and experimental procedures were carried out according to the guidelines of the Canadian Council on Animal Care (CCAC 1993). Relative humidity was 50-60% and temperatures ranged from 21 – 23 ºC. Normal
standard chow diet and water ad libitum were fed to the animals. Hepatocytes were isolated from rats as described by Moldéus and coworkers (Moldeus et al., 1978). Isolated hepatocytes (10^6 cells/mL) (10mL) were suspended in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continually rotating 50 mL round-bottomed flasks under 95% O₂ / 5% CO₂ conditions in a water bath maintained at 37 °C for 30 min (Moldeus et al., 1978). Then at various times (Figure 2.1), ROS was assayed using dichlorofluorescein diacetate (DCFD) and mitochondrial membrane potential (MMP) was assayed using rhodamine 123.

Figure 2.1. Isolation of hepatocytes and preparation and assays used. The liver was harvested from male Sprague Dawley rats. The hepatocytes were then isolated via collagenase perfusion. Next, the hepatocytes were added to rotating flasks. In each flask, the hepatocytes were put under different experimental conditions. Finally, at various time points, cell viability, ROS level and MMP status were assayed.
2.3 Cell viability

Cell viability was assessed microscopically via plasma membrane disruption determined by 0.1% w/v trypan blue exclusion test (Moldeus et al., 1978). The cells were allowed 30 min to acclimatize before initiating experiments. A cell viability of at least 80-90% had to be achieved before use. After this initial equilibration of period, cell viability was determined every 30 min during a 3 h incubation. GO and MGO was prepared immediately prior to use and were added to the hepatocytes before or after addition of protective agents. Pre-incubation with 1-bromoheptane was used to deplete hepatocyte GSH (Figure 2.2) (Khan and O’Brien, 1991). Its use is indicated in each table where it was used. Lethal concentrations of GO and MGO were also used to cause 50% cytotoxicity at 2 h (LD50, 2 h).

![Figure 2.2. Depletion of GSH with 1-bromoheptane. 1-bromoheptane reacts with GSH catalyzed by GST to form an irreversible conjugate, heptyl-s-glutathione. Glutathione (GSH); glutathione-s-transferase (GST); heptyl-s-glutathione (heptyl-S-GSH) (Adapted from Khan and O’Brien, 1991).](image-url)
2.4 Reactive oxygen species formation

Hepatocyte reactive oxygen species generation was determined by incubating DCFD with hepatocyte (Figure 2.3). DCFD entered the cell and was hydrolyzed to form non-fluorescent dichlorofluorescein (DCF). DCF reacted with ROS to form a highly fluorescent DCF which effluxed the cell (Possel et al., 1997). Samples (1 mL) were withdrawn at 45 and 90 min. These samples were centrifuged at 5000 x g for 1 min. The supernatant was aspirated. The cells were resuspended in H2O and 1.6 µM DCFD. The samples were incubated for 10 min at 37 °C. The samples were measured at 480 nm excitation and 520 emission wavelengths (Pourahmad and O’Brien, 2000).
Figure 2.3. Measurement of ROS generation using DCFD. DCFD enters the cell and is hydrolyzed to form a non-fluorescent DCF. DCF is oxidized by ROS to form a fluorescent DCF which effluxes the cell. Dichlorofluorescein-diacetate (DCFDA); dichlorofluorescein (DCF) (Adapted from Gomes et al., 2005).

2.5 Mitochondrial membrane potential assay

Rhodamine 123 accumulates selectively in mitochondria by facilitated diffusion. When the mitochondrial membrane potential is decreased, the amount of rhodamine 123 that enters the cell is decreased. Samples (0.5 mL) were withdrawn at 45 and 90 min and centrifuged at 5000 x g for 1 min. The supernatant was aspirated. The cell pellet was resuspended in 2 mL of Krebs-Henseleit buffer with 1.5 μM rhodamine 123 and incubated at 37 °C for 10 min. The
hepatocytes were separated by centrifugation and the amount of rhodamine 123 in the supernatant was measured fluorimetrically at 480 nm excitation and 520 nm emission wavelengths. The mitochondrial uptake of rhodamine 123 was calculated as the difference in fluorescence intensity between control and treated cells (Andersson et al., 1987).

2.6 Detection of protein carbonyl content of bovine serum albumin

Total protein bound carbonyl content of bovine serum albumin were measured using DNPH (Figure 2.4). Bovine serum albumin (2 mg/mL) was prepared in 100 mM sodium phosphate buffer, pH 7.4. Samples were incubated over a 6 day period. Time points were taken at 15, 60 and 120 min, and 1, 3 and 6 days. At each time point, 0.5 mL of sample was withdrawn and incubated with 0.5 mL of DNPH (0.1% w/v) in 2 N HCl for 1 h at room temperature in the dark. TCA (1 mL of 20% w/v) was added to stop the reaction and precipitate the protein. The sample was centrifuged at 5000 x g for 1 min. The supernatant was aspirated and DNPH was removed by washing the pellet with 0.5 mL of ethyl acetate:ethanol (1:1) solution three times. After washing, the pellets were dried with nitrogen and dissolved in 1 mL of 2 M Tris-buffered 8.0M guanidine-HCl, pH 7.2. The samples were read spectrometrically at 370nm (Hartley et al., 1997).
Figure 2.4 Detection of protein carbonyl content with 2,4-dinitrophenylhydrazone (DNPH).

Proteins with an amino group such as lysine and arginine can become modified by reactive dicarbonyls such as glyoxal. The modified protein is called a Schiff base. 2,4-dinitrophenylhydrazone can react with the Schiff base to form a conjugate that can be detected at 370 nm (Adapted from Hartley et al. 1997).

2.7 Determination of reactive dicarboxyls

The formation of dicarboxyls from fructose and dihydroxyacetone as a function of time under standard or oxidative stress conditions were determined using the Girard’s reagent T assay (Figure 2.5). Ten mM dihydroxyacetone, 10 mM fructose and Fentons reagent (200 µM Fe(II)/EDTA + 1 mM H₂O₂) stocks of 50 µL were added to 120 mM sodium phosphate buffer, pH 7.5, to a total of 1 mL reaction mixture and were incubated over a 6-day period. 50 µL of
reaction mixture samples were added to 950 µL of 120 mM sodium borate buffer, pH 9.3, at various time points at 15 min, 1 h, 2 h, day 1, day 3 and day 6. The mixture was vortexed and allowed 10 min to achieve equilibrium. 200 µL of this mixture was added to 800 µL of 100 mM Girard’s reagent T. The amount of dicarbonyl formation was detected spectrophotometrically at 326 nM (Voziyan et al., 2002; Mitchel and Birnboim, 1977). The concentration of dicarbonyl was determined using a standard curve.

![Chemical reaction diagram]

Figure 2.5. Detection of aldehydes or ketones with the Girard’s T reagent. The hydrazine, Girard’s T reagent, can react with aldehydes (R₁ or R₂ = H) or ketones (R₁ and R₂ = CHₓ) to form a hydrazone derivative that can be detected at 326 nm (Adapted from Johnson, 2007).

2.8 Statistical analysis

The statistical significance of non-hepatocyte and hepatocyte experiments was determined by one way analysis of variance (ANOVA), with post hoc Tukey’s analysis using SPSS. Values were considered statistically significant when P<0.05. Results if not indicated otherwise are from three independent experiments presented as mean±standard error of the mean (SEM).
1. Introduction

In diabetes mellitus patients, abnormal glucose metabolism leads to glucose intolerance and hyperglycemia. The excessive amount of sugars overwhelms the endogenous antioxidant and detoxifying systems. Under hyperglycemic conditions, the formation of toxic metabolites such as glyoxal and methylglyoxal increases (Shangari et al., 2003). These reactive dicarbonyls promote oxidative stress and can cause a number of cellular damages including covalent modification of amino and thiol groups of proteins to form advanced glycation endproducts (AGEs) (Ahmed, 2005; Thornalley et al., 1999). AGEs have been implicated in the pathogenesis of diabetic complications such as atherogenesis, nephropathy and cataractogenesis (O’Brien et al., 2005; Negre-Salvayre et al., 2008). Clinically, a number of therapeutic agents have been used to target the AGEs directly or indirectly. Aminoguanidine prevented AGE formation at an early stage by scavenging precursors such as glyoxal (Chen et al., 2004; Thornalley, 2003). Metformin is a biguanide compound that is currently used to control type 2 diabetes (Kiho et al., 2005). Metformin has also been shown to trap glyoxal (Peyroux and Sternberg, 2006). Other effective agents used included hydralazine, pyridoxamine, penicillamine, and N-acetyl-cysteine which also target reactive dicarbonyls (Mehta et al., 2009).

The consumption of fructose has increased significantly in the Western diet during the past three decades largely due to the introduction of high fructose corn syrup (HFCS) (Vos et al., 2008; Bray et al., 2004). Increased fructose consumption has been linked to many health concerns such
as metabolic syndrome and obesity. In particular, high fructose consumption leads to hepatic de novo lipogenesis, which subsequently contributes to increased visceral fat and insulin resistance, making fructose an important player in the pathogenesis of diabetes (Tappy et al., 2010).

Fructose and several of its metabolites can also form reactive dicarbonyls such as glyoxal, which carbonylates proteins and forms AGEs (Yang et al., 2011b). Methylglyoxal is formed from the metabolism of glucose and the metabolism of ketone bodies from acetone (Vander Jaqt et al., 2001). Glyoxal and methylglyoxal, the α-oxoaldehydes, are arginine-directed glycating agents, forming hydroimidazolones, which are quantitatively important AGEs with relatively short half-lives (12-60 days; Ahmed and Thornalley, 2007). The AGEs are involved in the macro and micro vascular complications in diabetic patients as they accumulate at the sites of vascular complications such as renal glomeruli, retina and peripheral nerves (Thornalley et al., 2003). AGEs can also exert their physiological effects by binding to cell surface receptors such as receptors of advanced glycation end products (RAGE), as well as the macrophage scavenger receptors ScR-II and CD-36 (Busch et al., 2010). AGE-RAGE interactions could also lead to oxidative stress conditions and radical formation as activation of RAGE indirectly activates pro-inflammatory transcriptional factor NF-κB and generates reactive oxygen species (reactive oxygen species) from activated NADPH oxidase (Schmidt et al., 2001; Wautier et al., 2001).

As previously demonstrated by this laboratory, fructose-induced hepatotoxicity increased more than 100-fold in the presence of a nontoxic concentration of H$_2$O$_2$ so as to mimic H$_2$O$_2$ levels formed by NADPH oxidase (NOX) released by activated immune cells such as neutrophils, eosinophils and macrophages (Lee et al., 2009). The increased hepatotoxicity was mainly due to the enhanced conversion of fructose and fructose metabolites to form glyoxal and also the
synergistic hepatotoxicity between glyoxal and H$_2$O$_2$ (Shangari et al., 2006; Yang et al., 2011a). Glyoxal can also be formed from the autoxidation of glyceraldehyde, glycoaldehyde and hydroxypyruvate in the absence of enzymes (Yang et al., 2011a). However, it is unclear whether the glyoxal formation from fructose and its metabolite dihydroxyacetone are enzyme-dependent. Understanding the relative contribution of autoxidation or enzymatic reaction to glyoxal formation could help in the development of more effective therapeutic agents for diabetes.

Various clinical studies have attempted to improve the clinical outcome of diabetic patients by introducing dietary and life style changes. A number of natural polyphenols have been demonstrated to have beneficial effects on diseases such as cancer, cardiovascular diseases and inflammation. Gallic acid is found in various plants and has been implicated in herb-based therapy for hyperglycemia (Gulati et al., 2012). Methyl gallate, ethyl gallate and propyl gallate are esters of the gallic acid group. Ethyl gallate and propyl gallate are added to foods containing unsaturated oil to prevent oxidation (2007; Mink et al., 2011).

Curcumin from turmeric and rutin from a variety of fruits and leaves have also been investigated (Kamalakkannan and Prince, 2006). Curcumin was shown to have anti-cancer capability through mechanisms which disrupt the mitochondrial membrane potential (Guo et al., 2012; Guo et al., 2012). The polyphenols have been shown to have reactive oxygen species-scavenging capability in addition to anti-cancer potential (Umadevi et al., 2012; Subudhi and Chainy, 2012; Kalaivani et al., 2011; Crispo et al., 2010; Criado et al., 2012).
The hepatoprotective (toxin and polyphenol added simultaneously) and rescue (a delay of 30 min before addition of polyphenols to toxin) ability of gallic acid and its gallate derivatives, rutin and curcumin against methylglyoxal and glyoxal have been investigated in physiological and GSH-depleted hepatocytes. In addition, reactive oxygen species formation and the mitochondrial membrane potential for each of the protective agents were analyzed. In this study, the polyphenols were ranked in their order of preventing hepatotoxicity, reactive oxygen species-scavenging capability and maintaining the mitochondrial membrane potential.

3. Scientific results

The purpose of these experiments was to first demonstrate that fructose and dihydroxyacetone were able to form reactive dicarbonyls that caused protein carbonylation under standard and oxidative stress conditions (Section 3.1). Next, several selected natural polyphenols were used as hepatoprotective agents against the reactive dicarbonyls (Section 3.2 – 3.4).

3.1 Autoxidation of fructose and dihydroxyacetone under standard or oxidative stress conditions

Dihydroxyacetone was readily autoxidized by Fenton’s reaction to form glyoxal and methylglyoxal in standard (37 °C, pH 7.4 buffer) and oxidative stress conditions (Fenton’s reaction) as shown in the bovine serum albumin protein carbonylation assay (Table 3.1) and the Girard Assay (Table 3.2). In the bovine serum albumin assay, dihydroxyacetone caused significant protein carbonylation in standard and in oxidative stress conditions compared to bovine serum albumin alone (Table 3.1). The Fenton’s control condition showed significant protein carbonylation. Fructose alone did not show significant protein carbonylation, and
addition of fructose to Fenton’s condition did not increase protein carbonylation when compared to the Fenton’s control condition (Table 3.1).
### Table 3.1
Carbonylation of serum albumin by dihydroxyacetone and fructose

<table>
<thead>
<tr>
<th></th>
<th>15 min</th>
<th>60 min</th>
<th>120 min</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA (2mg/mL)</td>
<td>5.2 ± 0.2</td>
<td>5.3 ± 0.1</td>
<td>5.5 ± 0.5</td>
<td>5.9 ± 0.3</td>
<td>5.3 ± 0.1</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>+Fentons</td>
<td>11 ± 1</td>
<td>12 ± 1</td>
<td>12 ± 1</td>
<td>21 ± 3</td>
<td>20 ± 4</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>+10mM DHA</td>
<td>13 ± 2</td>
<td>14 ± 2</td>
<td>14 ± 2</td>
<td>33 ± 1</td>
<td>29 ± 4</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>+Fentons</td>
<td>15 ± 3</td>
<td>22 ± 2</td>
<td>26 ± 1</td>
<td>76 ± 2</td>
<td>57 ± 2</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>+10mM Fructose</td>
<td>5.5 ± 0.3</td>
<td>5.7 ± 0.6</td>
<td>5.6 ± 0.1</td>
<td>7.0 ± 0.7</td>
<td>6.6 ± 1.0</td>
<td>6.1 ± 1.2</td>
</tr>
<tr>
<td>+Fentons</td>
<td>9.6 ± 1.6</td>
<td>12 ± 2</td>
<td>12 ± 2</td>
<td>16 ± 2</td>
<td>16 ± 2</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>

Fenton-induced oxidative condition generated with 200 μM FeII/EDTA + 1mM H₂O₂. Mean±SEM for three separate experiments are given.

*a* Significant as compared to control (p < 0.05);

*b* Significant as compared to 10mM DHA (p < 0.05);

*c* Significant as compared to 10mM Fructose (p < 0.05);

*d* Significant as compared to 10mM Fructose+Fentons (p < 0.05).
In the Girard assay (Table 3.2), dihydroxyacetone showed significant formation of dicarbonyl compounds, presumed to be glyoxal and methylglyoxal. Fructose showed statistically insignificant dicarbonyl formation. Fructose formed a little more dicarbonyl under Fenton’s-induced oxidative stress condition, but the amounts were not significant, as less than 0.02% of the 10 mM fructose was converted.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+Fentons</th>
<th>+10mM DHA</th>
<th>+10mM DHA + Fentons</th>
<th>+Fentons</th>
<th>+10mM Fructose</th>
<th>+10mM Fructose + Fentons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>60 min</td>
<td>120 min</td>
<td>Day 1</td>
<td>Day 3</td>
<td>Day 6</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.07 ± 0.02</td>
<td>0.08 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.07 ± 0.02</td>
<td>0.01 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>+Fentons</td>
<td>0.61 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.82 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.56 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.7 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.50 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>+10mM DHA</td>
<td>1.00 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.5 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.4 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>+Fentons</td>
<td>3.3 ± 0.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.3 ± 1.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>15 ± 1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>6.1 ± 0.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5.8 ± 0.8&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>4.2 ± 1.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>+10mM Fructose</td>
<td>0.11 ± 0.05</td>
<td>0.2 ± 0.1</td>
<td>0.24 ± 0.05</td>
<td>0.7 ± 0.7</td>
<td>0.3 ± 0.2</td>
<td>0.21 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>+Fentons</td>
<td>0.64 ± 0.04&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>0.7 ± 0.2&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>0.9 ± 0.1&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>2.2 ± 0.1&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>1.8 ± 0.2&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>1.2 ± 0.1&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Fenton-induced oxidative condition generated with 200 μM FeII/EDTA + 1mM H₂O₂; Control is 0.2 M sodium phosphate buffer pH 7.4. Mean±SEM for three separate experiments are given.

<sup>a</sup>Significant as compared to control (P < 0.05);
<sup>b</sup>Significant as compared to 10mM DHA (p < 0.05);
<sup>c</sup>Significant as compared to 10mM Fructose (p < 0.05);
<sup>d</sup>Significant as compared to 10mM Fructose + Fentons (p < 0.05).
3.2 Hepatoprotection and rescue against glyoxal with antioxidants in rat hepatocytes

The protective ability of antioxidants against glyoxal was assessed in freshly isolated rat hepatocytes. Glyoxal (5 mM, the LD_{50} for cells at 2 h of treatment) significantly increased hepatotoxicity and reactive oxygen species formation at 2 h of treatment (Table 3.3). The polyphenols showed significant hepatoprotection, decreased reactive oxygen species formation and improved maintenance of the mitochondrial membrane potential against 5 mM glyoxal. The rank order of hepatoprotection found for the polyphenols at 2 h and for preventing reactive oxygen species formation at 90 min was: rutin > alkyl gallates > gallic acid > curcumin (Table 3.3).
Table 3.3
Protective effects of polyphenols against glyoxal in rat hepatocytes.

<table>
<thead>
<tr>
<th>Treatment\Time Point</th>
<th>Cytotoxicity (% Trypan Blue taken up)</th>
<th>ROS (F.I. units)</th>
<th>% MMP</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 MIN</td>
<td>120 MIN</td>
<td>180 MIN</td>
<td>90 MIN</td>
</tr>
<tr>
<td>Control</td>
<td>26 ± 3</td>
<td>33 ± 3</td>
<td>40 ± 3</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>5 mM Glyoxal</td>
<td>38 ± 2</td>
<td>59 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>224 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Gallic Acid</td>
<td>24 ± 2</td>
<td>47 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75 ± 5</td>
<td>114 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Gallic Acid (30 min)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25 ± 3</td>
<td>41 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72 ± 6</td>
<td>135 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Methyl Gallate</td>
<td>25 ± 5</td>
<td>40 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>105 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Methyl Gallate (30 min)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23 ± 4</td>
<td>30 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>92 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Ethyl Gallate</td>
<td>28 ± 4</td>
<td>42 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78 ± 7</td>
<td>101 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Ethyl Gallate (30 min)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25 ± 2</td>
<td>35 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75 ± 6</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>0.2 mM Propyl Gallate</td>
<td>29 ± 5</td>
<td>47 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79 ± 6</td>
<td>94 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Propyl Gallate (30 min)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25 ± 3</td>
<td>40 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Rutin</td>
<td>26 ± 5</td>
<td>31 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>70 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Rutin (30 min)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23 ± 4</td>
<td>44 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77 ± 2</td>
<td>94 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 µM Curcumin</td>
<td>31 ± 3</td>
<td>48 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83 ± 6</td>
<td>136 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 µM Curcumin (30 min)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32 ± 3</td>
<td>42 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82 ± 8</td>
<td>134 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Isolated rat hepatocytes (10⁶ 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). Hepatotoxicity, ROS and MMP were determined as described in the methods section. Mean±SEM. for three separate experiments are given.

<sup>a</sup>Significant as compared to control (p < 0.05);
<sup>b</sup>Significant as compared to 5mM glyoxal (p < 0.05);
<sup>c</sup>Cells are incubated with glyoxal for 30 min before addition of antioxidants. The addition of glyoxal marks the initiation of the 3 h experiment period.
The rescue effect of the antioxidants was assessed by incubating freshly isolated rat hepatocytes with glyoxal for 30 min before the addition of antioxidants. The addition of glyoxal marked the initiation of the experiment (time zero). The polyphenols showed significant hepatoprotection, decrease in reactive oxygen species formation and improved mitochondrial membrane potential maintenance. The rank order for hepatoprotection at 2 h for hepatoprotection was: alkyl gallates > gallic acid = curcumin > rutin (Table 3.3). At 90 min, the formation of reactive oxygen species was decreased, but the rank order was: alkyl gallates > rutin > curcumin > gallic acid (Table 3.3). It appears that addition of the alkyl gallates after 30 min led to less cytotoxicity and lower reactive oxygen species formation than if the compounds were added initially. However, this was not true for rutin which led to increased cytotoxicity and reactive oxygen species formation.

3.3 Hepatoprotection and rescue by antioxidants against methylglyoxal in rat hepatocytes

The protective ability of antioxidants against methylglyoxal was assessed in freshly isolated rat hepatocytes (Table 3.4). Methylglyoxal (15 mM, LD50 for cells at 2 h of treatment) significantly increased cytotoxicity at 2 h. The polyphenols also significantly decreased cytotoxicity, reactive oxygen species formation and improved mitochondrial membrane potential maintenance. At 2 h the rank order for hepatoprotection was: rutin > curcumin > alkyl gallates > gallic acid. The ranking for prevention of reactive oxygen species formation at 90 min was: rutin > alkyl gallates > gallic acid > curcumin (Table 3.4).
Table 3.4
Protective effect of polyphenols against methylglyoxal in rat hepatocytes.

<table>
<thead>
<tr>
<th>Treatment\Time Point</th>
<th>Cytotoxicity (% Trypan Blue Taken)</th>
<th>ROS (F.I. units)</th>
<th>% MMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 MIN</td>
<td>120 MIN</td>
<td>180 MIN</td>
</tr>
<tr>
<td>Control</td>
<td>24 ± 2</td>
<td>34 ± 3</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>15mM Methylglyoxal</td>
<td>46 ± 4a</td>
<td>56 ± 5a</td>
<td>78 ± 5a</td>
</tr>
<tr>
<td>0.2 mM Gallic Acid</td>
<td>34 ± 6</td>
<td>43 ± 4b</td>
<td>49 ± 5b</td>
</tr>
<tr>
<td>0.2 mM Gallic Acid (30min)c</td>
<td>34 ± 4</td>
<td>41 ± 2b</td>
<td>46 ± 3b</td>
</tr>
<tr>
<td>0.2 mM Methyl Gallate</td>
<td>29 ± 5</td>
<td>34 ± 3b</td>
<td>51 ± 3b</td>
</tr>
<tr>
<td>0.2 mM Methyl Gallate (30min)c</td>
<td>28 ± 6</td>
<td>32 ± 4b</td>
<td>46 ± 4b</td>
</tr>
<tr>
<td>0.2 mM Ethyl Gallate</td>
<td>30 ± 4</td>
<td>34 ± 5b</td>
<td>54 ± 3b</td>
</tr>
<tr>
<td>0.2 mM Ethyl Gallate (30min)c</td>
<td>34 ± 3</td>
<td>33 ± 6</td>
<td>46 ± 5b</td>
</tr>
<tr>
<td>0.2 mM Propyl Gallate</td>
<td>35 ± 5</td>
<td>40 ± 4b</td>
<td>53 ± 4b</td>
</tr>
<tr>
<td>0.2 mM Propyl Gallate (30min)c</td>
<td>34 ± 6</td>
<td>38 ± 6</td>
<td>48 ± 6b</td>
</tr>
<tr>
<td>0.2 mM Rutin</td>
<td>25 ± 3b</td>
<td>30 ± 5b</td>
<td>47 ± 4b</td>
</tr>
<tr>
<td>0.2 mM Rutin (30min)c</td>
<td>30 ± 5</td>
<td>40 ± 4b</td>
<td>51 ± 5b</td>
</tr>
<tr>
<td>4uM Curcumin</td>
<td>36 ± 5</td>
<td>32 ± 5b</td>
<td>61 ± 6b</td>
</tr>
<tr>
<td>4uM Curcumin (30min)c</td>
<td>26 ± 3b</td>
<td>41 ± 6b</td>
<td>56 ± 4b</td>
</tr>
</tbody>
</table>

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). Hepatotoxicity, ROS and MMP were determined as described in method section. Mean±SEM. for three separate experiments are given.

aSignificant as compared to control (p < 0.05);
bSignificant as compared to 15mM methylglyoxal (p < 0.05);
cCells are incubated with methylglyoxal for 30 min before addition of antioxidants. The addition of methylglyoxal marks the initiation of the 3 h experiment period.
To assess the rescue effectiveness of the antioxidants against methylglyoxal, rat hepatocytes were pre-incubated with methylglyoxal for 30 min before adding the antioxidants. The polyphenols significantly decreased cytotoxicity, reactive oxygen species formation and improved the maintenance of mitochondrial membrane potential. The rank order of the rescue effect of antioxidants at 2 h for hepatoprotection was: alkyl gallates > rutin > gallic acid > curcumin (Table 3.4). At 90 min, the rank order for reactive oxygen species data was different from the rank order for hepatoprotection at 2 h. alkyl gallates > gallic acid = rutin = curcumin (Table 3.4).

Overall, the polyphenols were more hepatoprotective against methylglyoxal than against glyoxal. None of the polyphenols showed decreased cytotoxicity as compared to the cells incubated with the protective agents for the whole period. However, akin to glyoxal, this was not true for rutin which led to increased cytotoxicity and reactive oxygen species formation. The trends were similar to that for glyoxal. As the length of the alkyl side chain increased, cytotoxicity increased, reactive oxygen species scavenging ability increased, and mitochondrial membrane potential maintenance was improved.

3.4 Polyphenols protect against glyoxal and methylglyoxal in GSH-depleted hepatocytes

The rat hepatocytes were depleted of GSH via pre-incubation with 1-bromoheptane for 30 min. Glyoxal was added after the pre-incubation period and the addition of glyoxal marked the start of the 3 h experiment. In the GSH-depleted cells treated with glyoxal, all the polyphenols showed significant hepatoprotection, significant reactive oxygen species scavenging and improved mitochondrial membrane potential maintenance. The order of hepatoprotection at
2 h by the polyphenols was different than in non-GSH depleted hepatocytes: alkyl gallates = rutin > curcumin = gallic acid (Table 3.5). The rank order for preventing reactive oxygen species formation at 90 min was: rutin > alkyl gallates > gallic acid (Table 5). Curcumin was not effective at scavenging reactive oxygen species.
Table 3.5  
Protective effect of polyphenols against glyoxal and methylglyoxal in GSH-depleted rat hepatocytes.

<table>
<thead>
<tr>
<th>Treatment\Time Point</th>
<th>Cytotoxicity (% Trypan Blue Taken)</th>
<th>ROS (F.I. units)</th>
<th>% MMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 MIN</td>
<td>120 MIN</td>
<td>180 MIN</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH depleted hepatocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8mM Glyoxal</td>
<td>44 ± 4\textsuperscript{a}</td>
<td>62 ± 3\textsuperscript{a}</td>
<td>89 ± 2\textsuperscript{a}</td>
</tr>
<tr>
<td>0.2 mM Gallic Acid</td>
<td>34 ± 5</td>
<td>47 ± 2\textsuperscript{b}</td>
<td>63 ± 7\textsuperscript{b}</td>
</tr>
<tr>
<td>0.2 mM Methyl Gallate</td>
<td>34 ± 4</td>
<td>37 ± 2\textsuperscript{b}</td>
<td>58 ± 5\textsuperscript{b}</td>
</tr>
<tr>
<td>0.2 mM Ethyl Gallate</td>
<td>33 ± 4</td>
<td>41 ± 4\textsuperscript{b}</td>
<td>61 ± 2\textsuperscript{b}</td>
</tr>
<tr>
<td>0.2 mM Propyl Gallate</td>
<td>31 ± 2</td>
<td>45 ± 3\textsuperscript{b}</td>
<td>68 ± 4\textsuperscript{b}</td>
</tr>
<tr>
<td>0.2 mM Rutin</td>
<td>29 ± 3\textsuperscript{b}</td>
<td>42 ± 4\textsuperscript{b}</td>
<td>52 ± 2\textsuperscript{b}</td>
</tr>
<tr>
<td>4 µM curcumin</td>
<td>28 ± 4\textsuperscript{b}</td>
<td>47 ± 5\textsuperscript{b}</td>
<td>69 ± 5\textsuperscript{b}</td>
</tr>
<tr>
<td>3mM Methylglyoxal</td>
<td>54 ± 5\textsuperscript{a}</td>
<td>69 ± 4\textsuperscript{a}</td>
<td>84 ± 5\textsuperscript{a}</td>
</tr>
<tr>
<td>0.2 mM Gallic Acid</td>
<td>40 ± 6</td>
<td>50 ± 6\textsuperscript{c}</td>
<td>82 ± 5</td>
</tr>
<tr>
<td>0.2 mM Methyl Gallate</td>
<td>33 ± 4\textsuperscript{c}</td>
<td>40 ± 5\textsuperscript{c}</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>0.2 mM Ethyl Gallate</td>
<td>36 ± 5\textsuperscript{c}</td>
<td>44 ± 3\textsuperscript{c}</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>0.2 mM Propyl Gallate</td>
<td>39 ± 4</td>
<td>48 ± 5\textsuperscript{c}</td>
<td>76 ± 3</td>
</tr>
<tr>
<td>0.2 mM Rutin</td>
<td>33 ± 4\textsuperscript{c}</td>
<td>48 ± 2\textsuperscript{c}</td>
<td>82 ± 6</td>
</tr>
<tr>
<td>4 µM curcumin</td>
<td>30 ± 3\textsuperscript{c}</td>
<td>51 ± 2\textsuperscript{c}</td>
<td>79 ± 4</td>
</tr>
</tbody>
</table>

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). Hepatotoxicity, ROS and MMP were determined as described in the methods section. Mean ± SEM. for three separate experiments are given. In GSH-depleted cells, cells were pre-incubated with 1-bromoheptane for 30 min before addition of glyoxal or methylglyoxal and antioxidants.

\textsuperscript{a}Significant as compared to control (p < 0.05);
\textsuperscript{b}Significant as compared to 0.8mM glyoxal (p < 0.05);
\textsuperscript{c}Significant as compared to 3mM methylglyoxal (p < 0.05).
To assess the ability of antioxidants for protecting against methylglyoxal in GSH-depleted hepatocytes the same procedure as for glyoxal was used. The polyphenols significantly prevented-methylglyoxal-induced cytotoxicity, scavenged reactive oxygen species and improved mitochondrial membrane potential maintenance. The rank order for hepatoprotection at 2 h was the same as that for glyoxal (Table 3.5). At 90 min, the rank order for scavenging reactive oxygen species was similar to that for glyoxal, except that curcumin and gallic acid were able to scavenge reactive oxygen species.

4. Discussion of scientific results

Consumption of fructose has significantly increased over the past three decades as a result of the addition of HFCS to the diet (Vos et al., 2008; Bray et al., 2004). High fructose diets are associated with glucose intolerance, obesity, fatty liver, diabetes and non-alcoholic steatohepatitis (Yang et al., 2011a). Previously, fructose and its metabolites have been shown to generate glyoxal through enzymatic metabolism (Yang et al., 2011b; Lee et al., 2009; Yang et al., 2011a). Under oxidative conditions, it was determined that fructose cytotoxicity was significantly increased in hepatocytes (Yang et al., 2011b; Shangari and O’Brien, 2004). Dihydroxyacetone is part of the fructose metabolic pathway and can be further metabolized to form glyoxal and methylglyoxal (Yang et al., 2011b; Yang et al., 2011a; Seneviratne et al., 2012). Dihydroxyacetone and fructose metabolites have been shown to cause DNA damage and glycate human serum albumin in vitro (Seneviratne et al., 2012). However, whether dihydroxyacetone can autoxidize to glyoxal and methylglyoxal has not been investigated. This study investigated the autoxidation of fructose and dihydroxyacetone under standard and oxidative stress conditions. The Fenton’s reaction was used to generate oxidative stress
conditions by producing hydroxyl radicals (Feng et al., 2009). The bovine serum assay and the Girard-T assay were employed to investigate the ability of sugars to cause protein carbonylation and to form dicarbonyls, respectively.

In the bovine serum albumin assay, it was found that fructose did not cause protein carbonylation under standard conditions and with Fenton’s treatment, fructose produced less protein carbonylation than with the control Fenton’s condition (Table 1). Fructose has been shown to scavenge iron molecules, leading to decreased oxidative stress compared to the control Fenton’s condition (Spasojevic et al., 2009). The number of carbonyl groups formed per bovine serum albumin molecule varies depending on the physical and chemical environment (Chesne et al., 2006). In our experiments, dihydroxyacetone under Fenton’s condition generated a maximum of 2.5 carbonyl groups per bovine serum albumin molecule, and fructose under Fenton’s condition generated a maximum of 0.5 carbonyl groups per molecule. Under the Fenton’s condition alone, protein carbonylation occurred, but this was due to protein oxidation by the generated reactive oxygen species (Benoy and Beema, 2003).

In the Girard assay, it was determined that dihydroxyacetone autoxidized to dicarbonyl compounds under standard and oxidative stress conditions. The concentration of glyoxal decreased after day one in all treatments (Table 2), because at atmospheric conditions the half-life of glyoxal is one day (Atkinson, 1985). In contrast, fructose did not oxidize significantly to dicarbonyls with or without Fenton's reagent. It is important to note that the metabolites of fructose generate glyoxal and methylglyoxal enzymatically and non-enzymatically (Yang et al., 2011b). Our results differ from those of Manini et al.(Manini et al., 2006) in the amount of
fructose oxidation under iron-induced oxidative stress conditions (Manini et al., 2006). However the difference in the level of protein carbonylation and dicarbonyl formation for fructose was significantly less than that formed by dihydroxyacetone (Manini et al., 2006).

The protection with polyphenols against glyoxal and methylglyoxal was investigated in rat hepatocytes. Gallic acid and its related gallate derivatives are known to be strong antioxidant, anti-inflammatory, anti-mutagenic and anti-cancer compounds (Figure 3.1) (Umadevi et al., 2012; Kalaivani et al., 2011; Crispo et al., 2010; Atkinson, 1985).
Figure 3.1. Chemical structures of the polyphenols investigated. Gallic acid and the gallate derivatives and rutin can be found in tea leaves. Curcumin is the main compound found in the spice turmeric.

The antioxidative property of the gallate compounds is related to their lipophilicity (Umadevi et al., 2012; Atkinson, 1985). These gallate compounds also elicited cytotoxicity at high concentrations due to interaction with the mitochondrial membrane (Atkinson, 1985; Frey et al.,
Previous research demonstrated that the gallates with longer alkyl groups are toxic to hepatocytes due to their ability to cause mitochondrial uncoupling (Frey et al., 2007). However, the experiments conducted here showed little differences among the gallates. Presumably, this was due to the low concentrations of the polyphenols used. Rutin, the glycosidic form of quercetin, is a polyphenolic flavonoid with antioxidative and anticancer capabilities (Janbaz et al., 2002). Rutin is also a protector of mitochondrial function (Carrasco-Pozo et al., 2012). Curcumin is the principal curcuminoid of the Indian spice turmeric (Hsuuw et al., 2005). Curcumin is a potent antioxidant and anti-cancer compound (Guo et al., 2012). At high concentrations, curcumin is able to induce apoptosis both in cancer and primary cells (Guo et al., 2012; Hsuuw et al., 2005). The cytotoxicity of curcumin is likely due to its ability to uncouple the mitochondria, activate caspase-3 and activate the mitochondrial apoptotic pathway (Guo et al., 2012). Additionally, curcumin induces the release of cytochrome c from the mitochondria (Guo et al., 2012). At lower concentrations, curcumin demonstrated the ability to inhibit cytochrome c release and caspase 3 activation in mouse embryonic stem cells (Hsuuw et al., 2005). Also, curcumin can trap methylglyoxal at 1:1 ratios (Hu et al., 2012).

The level of hepatoprotection was determined with the trypan blue cytotoxicity assay, the measurement of reactive oxygen species and the measurement of mitochondrial membrane potential. The antioxidants significantly protected the rat hepatocytes against glyoxal under physiological conditions in terms of cytotoxicity, prevention of reactive oxygen species formation and maintaining the mitochondrial membrane potential (Table 3.3). The rankings are described in section 3.2 and 3.3. As the length of the alkyl chains increased, the polyphenols demonstrated less hepatoprotection due to the disruption of mitochondrial function. In contrast,
as the length of the alkyl chain increased, reactive oxygen species scavenging ability also increased (Umadevi et al., 2012; Atkinson, 1985). Curcumin was less protective against glyoxal than against methylglyoxal.

Furthermore, the rescuing ability of each polyphenol was assessed. Glyoxal and methylglyoxal were pre-incubated for 30 min before addition of the antioxidants. The order of hepatoprotection was similar for glyoxal and methylglyoxal, but the rescue effect by polyphenols was larger for glyoxal (Table 3.3 and 3.4). A plausible explanation is that the antioxidants do not scavenge glyoxal or methylglyoxal directly but rather target downstream effects such as generation of reactive oxygen species or disruption of the mitochondrial membrane potential (Umadevi et al., 2012; Kalaivani et al., 2011; Crispo et al., 2010; Janbaz et al., 2002; Chen et al., 2010; Khan and O’Brien, 1991). Another explanation is that the polyphenols have innate hepatotoxicity leading to increased hepatotoxicity when hepatocytes are treated for a longer period (Nakagawa and Tayama, 1995). Curcumin was an exception in the order of hepatoprotection, where curcumin demonstrated greater hepatoprotection against methylglyoxal than glyoxal. Curcumin is a methylglyoxal scavenger with a 1:1 ratio of scavenging ability (Chen et al., 2010). Methylglyoxal is trapped between the two keto-carbon groups at C10 (Hu et al., 2012). Another exception in the order of hepatoprotection was rutin, which demonstrated less hepatoprotection when rescuing against glyoxal and methylglyoxal (Table 3.3 and 3.4). Although rutin is an antioxidant, metabolites of rutin such as quercetin also elicit antioxidative properties (Inoue et al., 1995). The results of this study have shown that the metabolites of rutin could be better antioxidants than rutin. Thus, simultaneously adding rutin and the toxins allowed rutin to be converted into its more potent metabolites (Pashikanti et al., 2010). Curcumin consistently
placed last in the rankings as well. Gallic acid was found to be cytotoxic and does not follow the trend of increasing alkyl chain length and hepatotoxicity. Gallic acid has three adjacent phenolic hydroxyl groups that are linked with its hepatotoxicity (Inoue et al., 1995).

The effect of GSH-depletion and the protection against glyoxal and methylglyoxal with the antioxidants was investigated. 1-Bromoheptane was used as the agent to deplete GSH irreversibly (Khan and O’Brien, 1991). The depletion of GSH promotes oxidative stress and compromises the detoxification of many electrophilic compounds, including glyoxal and methylglyoxal (Khan and O’Brien, 1991). This study demonstrated the antioxidant ability of the polyphenols. The polyphenols reduced oxidative stress and alleviated some hepatotoxicity in GSH-depleted hepatocytes. Hepatotoxicity was greater in comparison with hepatocytes without GSH depletion because GSH is a co-factor for glyoxalase, which detoxifies glyoxal and methylglyoxal (Vander Jaqt et al., 2001).

This study demonstrated the autoxidation of dihydroxyacetone into dicarbonyls and the inability of fructose to autoxidize. Furthermore, a variety of polyphenols were investigated for their protective and rescuing effect against dicarbonyls in rat hepatocytes. In the future, there will be investigations on the protective abilities of the polyphenols to determine if these mechanisms are enzyme-dependent. Understanding, their mechanisms of protections of polyphenols may lead to the production of novel therapeutic agents that targets the early AGE pathways or downstream effects of AGEs. The development of these agents can lead to treatment of various illnesses related to the metabolic syndrome.
4.1 General discussion

A further in-depth discussion of the findings mentioned above will be provided. The formation of dihydroxyacetone will be discussed (sub-section 5.1.1). Next, some of the roles and uses of dihydroxyacetone will be mentioned (sub-section 5.1.2). Finally, the mechanisms of protection by the natural polyphenols will be discussed (sub-section 5.1.3).

4.1.1 Dihydroxyacetone

The Western diet contains low amounts of greens, high amounts of fatty foods including meat or dairy products, and high amounts of sweets (Maghsoudi and Azadbakht, 2012). Increasingly, fructose, in the form of HFCS, has replaced the main sweetener, sucrose, in this type of diet (Anderson, 2007). Fructose has been associated with and demonstrated to cause metabolic syndrome, a collection of diseases including glucose intolerance, obesity, fatty liver, and sometimes non-alcoholic steatohepatitis in various human and animal studies. Many of these health concerns are related to the formation of AGEs, which originate from sugars and their metabolites. Fructose and its metabolites are highly capable of forming GO and MGO in vitro (Abordo et al., 1999; Banach et al., 2009). However, the activity of each of its metabolites, especially DHA, has not been studied before. Thus, it is one of the focuses of this study.

Fructose metabolism mainly takes place in the liver. Thus, the investigations were on isolated rat hepatocytes. Fructose and DHA can be metabolized into the reactive dicarbonyls, GO and MGO. Therefore, their ability to cause protein oxidation of carbonylation and the extent of their
autoxidation to form GO and MGO were carried out in a protein and chemical system respectively. In these cell free systems, it was found that fructose did not significantly autoxidize or cause protein carbonylation or autoxidation under physiological conditions (pH 7.4, 37°C). However, under oxidative stress condition (Fenton’s reaction), fructose significantly autoxidized to give significant protein carbonylation and form reactive dicarbonyls. When fructose was compared with DHA, there was a significant difference in the autoxidation rate under both conditions. This investigation found that DHA caused more protein carbonylation and was more readily autoxidizable in comparison to fructose. DHA was a more potent toxic agent than fructose.

4.1.2 Roles and uses of dihydroxyacetone

DHA is a triose sugar and is generated from fructose-1-phosphate and fructose-1,6-diphosphate catalyzed by aldolase B activity (Seneviratne et al., 2012). DHA and DHAP are interconvertible by a phosphatase and kinase enzyme (Slepokura and Lis, 2010). DHA has a wide array of applications (e.g. sunless tanning agent, antifungal and was an antidote for cyanide poisoning).

DHA (1.6-50 mg/mL) has anti-fungal properties at the same concentrations found in artificial suntan lotions (Stopiglia et al., 2011). DHA is used topically, where it reacts in the stratum corneum, thus, minimizing systemic absorption (Stopiglia et al., 2011). DHA can also act as an antidote for cyanide poisoning. Cyanide can form from metabolism of drugs and is found in smoke. Cyanide is highly nucleophilic and can react with carbonyl groups such as aldehydes and ketones to form less toxic cyanohydrin intermediates (Bhattacharya and Tulsawani, 2008). Trapping cyanide restores cellular respiration and ATP levels.
DHA was approved by the Food and Drug Administration (FDA) for use as an additive (5-15% w/w) in sunless tanning cosmetic products (Blau et al., 1960). In skin proteins, DHA causes the formation of AGEs which produces a brown pigmentation (Petersen et al., 2004). The keto group of DHA reacts with amino groups on proteins. Some of the amino acids that DHA can react with are glycine, proline, serine, asparagine and threonine (Ahmed et al., 2003). DHA is also genotoxic and can modify DNA, especially the nucleotide guanosine-5-triphosphate (GTP), forming various AGEs (Seneviratne et al., 2012). The non-enzymatic glycation of DNA can also decrease the stability of glycosidic bonds, leading to the depurination of DNA and creates mutagenic sites (Petersen et al., 2004). In cultured epidermal keratinocytes, DHA was demonstrated to cause DNA damage, cell cycle blockage and apoptosis (Petersen et al., 2004). In patients with triosephosphate isomerase (TPI) deficiency, DHA is found at high concentration and is viewed as the cause of TPI deficiency morbidity (Valentine et al., 1966). TPI deficiency is associated with neuromuscular disorders and chronic hemolytic anemia (Ahmed et al., 2003). GSH levels were decreased and there were marked levels of oxidative stress, suggesting MGO was associated with TPI deficiency (Karg et al., 2000). MGO was spontaneously formed from the degradation of DHA. Hence, DHA is associated with various complications. After confirming the ability of fructose and DHA to form reactive dicarbonyls, the next step was to find protective agents.

4.1.3 Polyphenols as protective agents

Natural polyphenols are a class of phytochemicals with antioxidant properties. There are several classes of polyphenols and they are found in foods consumed on a daily basis. Their beneficial
effects include lowering the risk of cancer, cardiovascular diseases, hypertension, neurodegenerative diseases and stroke (Wolfe et al., 2003). The polyphenols investigated were natural polyphenols (found in the original source) or were food additives. Foods containing fats and oil can undergo oxidation, leading to a loss of nutritional value and changes in their chemical composition. For example, formation of AGEs causes discoloration in fruits (Garrido et al., 2012). Lipid oxidation can initiate a free radical chain reaction. Various sources such as heat, light, singlet oxygen, ionizing radiation, transition-metal catalysis with copper, iron or manganese ions, can cause lipid oxidation (Garrido et al., 2012). Antioxidant additives, given E-numbers from E300 to E385 by the European Food Safety Authority, can delay, retard or prevent deterioration due to oxidation when present at low concentrations. Gallic acid derivatives (E310 – E312) are added to protect edible fats, vegetable oils and salad dressings from turning rancid due to oxidation (Garrido et al., 2012). Gallic acid derivatives are alkyl esters of gallic acids, which differ from each other in their side chains. Some gallic acid derivatives, such as propyl gallate, octyl gallate and dodecyl gallate, are used in foods, cosmetics, and pharmaceutical products (2007). The activity of these phenolic antioxidants depends on the structural features such as O-H bond dissociation energy, resonance delocalization of the phenoxy radical and steric hindrance from substituents on the aromatic ring (Garrido et al., 2012). For example, esterification with n-alkyl alcohols enhances hydrophobicity and improves antioxidant properties. The phenolic acids act as an antioxidant by donating the hydrogen atom on the phenolic group to free radicals, thereby trapping free radicals and preventing or minimizing the propagation of the chain reaction (Garrido et al., 2012). Thus, gallic acid and propyl gallate act as antioxidants by having numerous and different phenolic groups on the aromatic ring. The free radical scavenging potential is dependent on the number and location of free hydroxyl groups in
the aromatic ring (Brewer, 2011). The carboxyl group on gallic acid is electrophilic and has a negative influence on the hydrogen donating ability of hydroxyl groups in the aromatic ring. Thus, this group is usually esterified to improve antioxidant properties. Also, esterification improves the lipophilicity of phenolic compounds and allows their use in organic media (Garrido et al., 2012).

Phenolic acids act as free radical trapping reagents. Flavonoids, such as rutin, act as free radical scavengers (Brewer, 2011). Flavonoids are a 15-carbon flavan compound (C$_6$C$_3$C$_6$) arranged in three rings (A, B and C). Each class of flavonoids differs in the level of saturation of the C ring. Within the same class, the substitution pattern of ring A and B differs, which influences the phenoxy radical stability and antioxidant property (Brewer, 2011). Rutin is a flavonol and the B ring substitution pattern is especially important in determining its antioxidant property (Wojdylo et al. 2007). Flavonoids with multiple hydroxyl groups are more effective antioxidants. The free radical scavenging potential is dependent on the pattern (number and location) of free hydroxyl groups. Additionally, flavonoids can also decrease the effectiveness of transition metals at promoting oxidation by donating hydrogen atoms.

Curcumin is a polyphenol derived from turmeric spice and is used as food color, spice and the prevention and therapy of many diseases. The compound contains two ferulic acid residues joined by a methylene bridge (Gupta et al., 2013). The antioxidant property of curcumin derives from the hydrogen donating phenolic hydroxyl groups (Zingg et al., 2013). Curcumin can also reduce the generation of free radicals by chelating Fe$^{2+/3+}$, Cu$^{2+}$ and Mn$^{2+}$. Curcumin is a pleiotropic molecule that modulates multiple signaling pathways (Gupta et al., 2013). Due to the
structure of curcumin, it can interact with proteins at the molecular level in various ways such as van der Waals interaction, hydrogen bonding, metal chelation and covalent binding (Gupta et al., 2011). This allows curcumin to modulate various signaling pathways. The phenyl rings can participate in van der Waals interactions with aromatic amino side chains (Gupta et al., 2011). The phenolic and carboxyl group can hydrogen bond with macromolecules (Gupta et al., 2011). Curcumin can also undergo keto-enol tautomerization. In the predominant enol form, curcumin can chelate transition metals (Baum and Ng, 2004). The enol form also allows the midsection of curcumin to accept and donate hydrogen bonds (Gupta et al., 2011). Lastly, curcumin has also been found to covalently bind to nucleophilic cysteine sulfhydryls and selenocysteine moiety (Fang et al., 2005; Marcu et al., 2006). Curcumin can modulate or prevent inflammation by antagonizing pro-inflammatory signals and gene expression. Curcumin can suppress NF-κB and STAT3, leading to the downregulation of TNF-α and IL-6 (Zingg et al., 2013). Curcumin can also bind directly to TNF-α, cyclo-oxygenase I (COX-1) and II (COX-2), NADPH oxidase, xanthine oxidase and iNOS, thereby indirectly decreasing ROS generation (Zingg et al., 2013; Gupta et al., 2011). Though curcumin has a low bioavailability, it may bind to carrier proteins to increase its bioavailability (Gupta et al., 2011). In a 6-month study, human subjects consumed curcumin 1-4g/day and the plasma concentration was 490 nM (Schaffer et al., 2011). Also, curcumin is rapidly glucuronidated and sulfated or is converted to numerous metabolites. Tetrahydrocurcumin, a metabolite of curcumin, has a much higher antioxidant activity than curcumin (Zingg et al., 2013).

The protective effects of the polyphenols against GO and MGO were investigated because GO and MGO have different toxicities and were detoxified by different systems. In the GSH-
depleted hepatocytes, the investigations found that hepatoprotection against GO and MGO was overall less than hepatoprotection under control conditions. Additionally, it was found that hepatoprotection against GO was generally better than against MGO. Depleting the GSH compromised the hepatocytes’ ability to detoxify GO and MGO. In addition, there should be differences in hepatoprotection against GO and MGO as discussed below.

The glyoxalase system detoxifies GO to glycolate and MGO to D-lactate (Abordo et al., 1999). However, the glyoxalase system detoxifies the two dicarbonyls to different extents. It is generally viewed that MGO is mostly detoxified by glyoxalase I, which catalyzes hemithioacetal, formed spontaneously from the MGO-GSH complex, to S-D-lactoylglutathione (Thornalley, 1990).

MGO binds to the cysteine of GSH while GO binds to the α-NH₂ group of the glutamate residue (Vander Jagt et al., 2001). MGO and GO react with GSH in a similar fashion; however, their metabolism by glyoxalase differs (Yang et al., 2011b). GO is likely a poor substrate for glyoxalase I as shown by Baba et al. (Baba et al., 2009). Instead, GO is metabolized by mitochondrial aldehyde dehydrogenase (ALDH2), another major carbonyl detoxifying enzyme (Kraemer and Dietrich, 1968). ALDH2 metabolizes GO and MGO, however, using ALDH2 inhibitors, it was demonstrated that GO relies on ALDH2 for detoxification while MGO relies on the glyoxalase system for detoxification (Abordo et al., 1999).
4.2 Limitations

These investigations show that the polyphenols were effective hepatoprotectors against GO and MGO under physiological and GSH-depleted conditions. The techniques used in this experiment allowed rapid assessments of cytotoxicity, ROS levels and MMP status. However, the hepatocyte suspension only allows the hepatocyte to survive for a few hours. The technique does not allow for studying long-term effects of the polyphenols on hepatocyte survivability. Hence, this technique only allows for the investigation of hepatoprotective effects of polyphenols on the acute toxicity of GO and MGO. Additionally, the long-term toxicity of GO and MGO on hepatocytes is unclear.

Another limitation to the study is that hepatocytes were exposed to a single polyphenol for each condition. However, the gut microflora converts the original compound to various metabolites. The investigation could not determine the metabolic potential of the gut microflora. Thus, the investigation did not determine the interaction between the original compound and their metabolites.

Lastly, the experiments were done in an in vitro setting and do not study the impact of the polyphenols in a systemic setting. An animal trial would allow investigators to study the effects of the polyphenols in each of the major organs. It would also allow researchers to study the bioavailability of the compounds.
4.3 Future perspectives

Several polyphenols were selected for future investigations. These are ferulic acid/ferulaldehyde, isoeugenol, rutin metabolites and quercetin. Each sub-section provides a rationale for the polyphenols being selected (Section 5.3.1 – 5.3.4). In the final sub-section, a future study plan involving an animal model will be described.

4.3.1 Ferulic acid

Polyphenols undergo various biotransformations upon entry into the body. Therefore, it is important to study the biological effects of polyphenol metabolism, which may differ from the original polyphenol. Ferulic acid is a water soluble endproduct of polyphenol degradation. It is found at high concentration in human urine upon consumption of chocolate and red wine (Rios et al., 2003). The antioxidant property of ferulic acid can also vary with different carbon side chain groups.

Ferulic acid is found in seeds and leaves in its free form or is covalently linked to biopolymers. Ferulic acid has three structural features that contribute to its antioxidant property. The electron donating groups on the benzene ring (methoxy and hydroxyl groups) give the compound the ability to terminate free radical chain reactions. The carboxylic group with an adjacent unsaturated C=C bond also provides an additional attack site for free radicals. Lastly, the carboxylic group can also bind to the lipid bilayer and act as protection against lipid peroxidation. The phenolic group and the unsaturated side chain can form stabilized resonance phenoxy radicals. The phenoxy radical cannot start a chain reaction; however, curcumin can be
formed upon collision and condensation with another ferulic acid radical (Srinivasan et al., 2007).

Ferulic acid has a higher bioavailability than other flavonoids and monophenolics (Srinivasan et al., 2007). Ferulic acid stays in the blood longer than vitamin C and therefore would be expected to detoxify free radicals more readily (Srinivasan et al., 2007). In enterocytes, ferulic acid is readily conjugated and is released into the circulation. Other investigations showed that ferulic acid has anti-inflammatory and anti-cancer properties. Ferulic acid can decrease oxidative stress in the β-pancreatic cells and allow the cells to proliferate and secrete insulin (Srinivasan et al., 2007). Ferulic acid may also regulate intracellular pathways, reduce proliferation and induce apoptosis in cancer cells (Beecher, 1998). Ferulic acid is also extensively studied for its effect in modulating the central nervous system (Lee et al., 2013). Ferulaldehyde, a ferulic acid derivative, has been studied for its anti-inflammatory properties. Ferulaldehyde was shown to decrease the levels of pro-inflammatory cytokines such as TNF-α and IL-6 in the lipopolysaccharide-induced septic shock murine model (Radnai et al., 2009). Additionally, ferulaldehyde was shown to inhibit the activation of NF-κB.

4.3.2 Isoeugenol

Isoeugenol is a constituent of plants used as a spice based on its odour and anti-septic because of its detergent-like properties (Chang et al., 2002; Tai et al., 2002). Isoeugenol also has anti-inflammatory properties because it can inhibit lipopolysaccharide-induced expression of inducible nitric oxide synthase and cyclooxygenase in macrophages (Li et al., 2006). Additionally, isoeugenol inhibits iron-mediated lipid peroxidation and copper-dependent LDL
oxidation (Ito et al., 2005). Isoeugenol has electron donating groups that increase the ease of hydrogen atom abstraction. This allows isoeugenol to act as a better antioxidant.

Ferulic acid and ferulaldehyde have electron withdrawing groups have the opposite effect (Nenadis et al., 2003). Some of the preliminary results of ferulic acid and its derivatives are shown in the appendix. The results warrant further investigation into their antioxidant properties under oxidative stress and GSH-depleted conditions.

4.3.3 Rutin metabolites

Rutin is a polyphenol with low bioavailability. It is known that little or no rutin is absorbed at all because of its extensive metabolism by gut microflora (Pashikanti et al., 2010). The rutin metabolites investigated are 3,4-dihydroxyphenylacetic acid, homovanillic acid and quercetin. These metabolites inhibit the formation of AGEs. Metabolites with vicinal dihydroxyl groups such as 3,4-dihydroxyphenylacetic acid are also potent in preventing AGE formation (Pashikanti et al., 2010). Compounds containing vicinyl dihydroxyl group inhibit the autoxidation of glucose (Cervantes-Laurean et al., 2006). They also show the ability to chelate iron and copper and prevent the catalyzed production of initiating radical species (Cervantes-Laurean et al., 2006). However, metabolites with monohydroxyl groups such as homovanillic acid are not as effective as antioxidants (Pashikanti et al., 2010). These metabolites can trap and inactivate MGO and GO via condensation of adjacent phenolic hydroxyl groups with dicarbonyls to form hemiacetal intermediates, which can further react to form acetal (Pashikanti et al., 2010).
4.3.4 Quercetin

The results also warrant further investigation of their protective capability under oxidative stress and GSH-depleted conditions. Quercetin is found in a variety of fruits, vegetables and beverages. In foods, quercetin is glycosylated and requires deglycosylation for its absorption (Russo et al., 2012). Quercetin has been associated with the prevention and therapy of cardiovascular disease and cancer. Metabolism of quercetin occurs in enterocytes and hepatocytes. Biotransformation occurs in the colon and absorption is influenced by gut microflora (Russo et al., 2012). Quercetin undergoes more biotransformation than rutin since rutin has a hydrophilic sugar moiety that impedes its access into cells and enzyme active sites (Vacek et al., 2012). Hence, a plasma level of quercetin rarely exceeds 1 μM. Preliminary results of these rutin metabolites are shown in the appendix.

4.3.5 In vivo studies

Lastly, the investigations shown here compared the antioxidant and cytoprotective capabilities of polyphenols in vitro. The results warrant further investigation in an in vivo setting. An animal model for diabetes will allow the researcher to investigate the efficacy of these compounds in a systemic setting. A screening process involving similar assays mentioned in this thesis can be used to discover highly effective polyphenols. Then, a comparative study using a diabetic rat model (induced by streptozotocin) between a control and a polyphenol-enriched diet can be used to study the positive effects of the polyphenols. The endpoint of this study is lower AGE levels in the major organs and blood. Blood samples can be taken throughout the experimental period to track differences in AGE levels.
There are a myriad of natural polyphenolic compounds that still await investigation. The few compounds investigated in this thesis are common dietary polyphenols. In the future, these polyphenols may be of use in clinical practice for various treatments.


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Chesne, S., Rondeau, P., Armenta, S., and Bourdon, E. 2006. Effects of oxidative modifications induced by the glycation of bovine serum albumin on its structure and on cultured adipose cells. *Biochimie*. **88**: 1466-477.


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### Table 5.1
Protective effect of polyphenolic metabolites against glyoxal in rat hepatocytes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytotoxicity (%) Trypan Blue Taken</th>
<th>ROS (F.I. units)</th>
<th>% MMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 MIN</td>
<td>120 MIN</td>
<td>180 MIN</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5mM Glyoxal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 mM Rutin</td>
<td>31 ± 3</td>
<td>38 ± 1</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>0.2 mM Quercetin</td>
<td>29 ± 4</td>
<td>34 ± 2</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>0.2 mM 3,4-Dihydroxyphenylacetic acid</td>
<td>33 ± 3</td>
<td>40 ± 3</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>0.2 mM Homovanillic acid</td>
<td>30 ± 2</td>
<td>40 ± 3</td>
<td>65 ± 2</td>
</tr>
<tr>
<td>0.2 mM Ferulaldehyde</td>
<td>27 ± 1</td>
<td>42 ± 4</td>
<td>59 ± 2</td>
</tr>
<tr>
<td>0.2 mM Ferulic acid</td>
<td>24 ± 3</td>
<td>41 ± 2</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>0.2 mM Isoeugenol</td>
<td>36 ± 3</td>
<td>40 ± 2</td>
<td>51 ± 2</td>
</tr>
</tbody>
</table>

Isolated rat hepatocytes (10⁶ 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). Hepatotoxicity, ROS and MMP were determined as described in method section. Mean±SEM, for three separate experiments are given.

aSignificant as compared to control (p < 0.05);
bSignificant as compared to 15mM methylglyoxal (p < 0.05).
### Table 5.2
Protective effect of polyphenolic metabolites against methylglyoxal in rat hepatocytes.

<table>
<thead>
<tr>
<th>Treatment\Time Point</th>
<th>Cytotoxicity (% Trypan Blue Taken)</th>
<th>ROS (F.I. units)</th>
<th>% MMP</th>
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<tr>
<td></td>
<td>60 MIN</td>
<td>120 MIN</td>
<td>180 MIN</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15mM Methylglyoxal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 mM Rutin</td>
<td>23 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40 ± 3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>55 ± 3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Quercetin</td>
<td>20 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34 ± 4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM 3,4-Dihydroxyphenylacetic acid</td>
<td>22 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34 ± 3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Homovanillic acid</td>
<td>24 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38 ± 2&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Ferulaldehyde</td>
<td>25 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35 ± 1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>48 ± 3&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
<td>0.2 mM Ferulic acid</td>
<td>23 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32 ± 3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>41 ± 4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 mM Isoeugenol</td>
<td>21 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40 ± 4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
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</table>

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). Hepatotoxicity, ROS and MMP were determined as described in method section. Mean±SEM, for three separate experiments are given.

<sup>a</sup>Significant as compared to control (p < 0.05);

<sup>b</sup>Significant as compared to 15mM methylglyoxal (p < 0.05).
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