Metabolism and bioactivity of catechins

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Thesis submitted in partial fulfilment of the requirements for the degree

of Doctor of Philosophy

Agriculture, food, and rural development, AFRD

May 2015

Acknowledgements

There are a lot of people I'd like to thanks for making this possible. Firstly I'd like to start by thanking Dr Georg Lietz for allowing me to complete a PhD under his supervision, for his support, guidance, and extreme patience. My gratitude also goes to Dr Martin Foltz at Unilever, Professor Mark Birch-Machin, and Dr Edward Okello for their inputs and help throughout these years. I've learnt a lot since I've been here so thanks again. Thank you for all the opportunities that you've given me while I've been under your wing.

I also want to thank everyone who has contributed directly or indirectly to me completing the thesis, colleagues and friends alike. Especially Christine, Anthony, Chris, Boudewijn, who helped and taught me how to use all the weird and wonderful stuff in the lab. Thanks to all my fellow PhD students who have had to put up with me and my humour for so long, I know you'll miss me. I would also like to extend a big thank you to the people in A5280 in Vlaardingen for your kindness and friendship while I was in the Netherlands.

Of course I can't leave my family out otherwise I'll never hear the end of it, so thanks for all the support these last 4 years even though you probably never knew what I was doing or where I was.

Finally the funders of this studentship, Unilever and the BBSRC are gratefully acknowledged for the financial support.

Abstract

Green tea is widely consumed worldwide, second only to water. Its health effects have been attributed to its polyphenolic catechin content, which accounts for a significant percentage of its dry weight. Epidemiological studies have observed beneficial health effects of green tea catechins against some cancers, cardiovascular diseases, and neurodegenerative diseases. However most studies have also been carried out in vitro where the concentrations of catechins used are not obtainable in the human body. The in vivo situation is very complicated and relies on favourable absorption, metabolism, and excretion of the catechins. In this thesis it is reported, the potential health benefits of green tea catechins in tissues which are highly and most often in contact with catechins, the oral cavity and the intestine. In experiments modelling the oral cavity the four major catechins were extensively absorbed into H400 cells, and showed significant protection at physiological concentrations against H_2O_2 induced mitochondrial DNA damage. Thereafter the *in vitro* metabolism of catechins were measured using various intestinal and liver enzyme fractions. It was shown, that in these systems, glucuronidation was the main metabolism pathway. These enzyme fractions also provided mixtures of metabolites which were used to measure their comparative bioactivities in relation to their parent compounds. This was done via the assessment of their protective effects against H_2O_2 induced mtDNA damage in human intestinal cells. The metabolites were shown to be significantly bioactive; however the majority of the metabolites had lower bioactivities than the parent compound. In the final study the aim was to inhibit the glucuronidation of catechins to aid in the development of strategies to enhance catechin bioavailability. The two compounds chosen have previously been shown to inhibit intestinal glucuronidation. In this study it was determined that both piperine and quercetin significantly inhibited the metabolism of the catechins in intestinal microsomes. These studies taken together give a platform for future studies to be carried out which will be more detailed and rigorous.

List of Abbreviations used

4′-MeEGC	4′-*O*-methyl-(−)-Epigallocatechin
4"-MeEGCG	4"-*O*-methyl-(−)-Epigallocatechin gallate
4′, 4"-DiMeEGCG	4′, 4"-di-*O*-methyl-Epigallocatechin gallate
ABC	ATP-binding cassette
ADME	Absorption, distribution, metabolism, and excretion
ATP	Adenosine triphosphate
bp	Base pairs
CIB	Cytoplasmic isolation buffer
C_{max}	Peak plasma concentration
COMT	Catechol-*O*-methyl transferase
C_t	Cycles to threshold
Da	Daltons
DCFH-DA	2', 7'-Dichlorodihydrofluorescin-Diacetate
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
EC	Epicatechin
ECG	Epicatechin gallate
EGCG	Epigallocatechin gallate
EGCG—Gluc	EGCG glucuronide
EGC	Epigallocatechin
ETC	Electron transport chain
FBS	Foetal bovine serum
GI tract	Gastro intestinal tract
HPLC	High-performance liquid chromatography

HPLC-DAD	HPLC-diode array detection
HPLC-MS	HPLC-mass spectrometry
H_2O_2	Hydrogen peroxide
IV	Intravenous
K_m	Substrate concentration at half V_{max}
kV	Kilo volts
mg	Milligrams
$MgCl_2$	Magnesium chloride
MIB	Membrane isolation buffer
µL	Microlitre
µg	Microgram
µM	Micromolar
mM	Millimolar
MCT	Monocarboxylate transporter
mRNA	Messenger RNA
MRP	Multidrug resistance-associated proteins
MtDNA	Mitochondrial DNA
m/z	Mass to charge ratio
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
nM	Nano molar
nm	nano meter
OSCC	Oral squamous cell carcinoma
OXPHOS	Oxidative phosphorylation
PAPS	Adenosine 3’-phosphate 5’-phosphosulfate lithium salt hydrate

PBS	Phosphate buffered saline
PGP	P-glycoprotein
pmol	Pico mole
ppm	parts per million
PPO	Polyphenol oxidase
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT- PCR	Reverse transcription polymerase chain reaction
SAM	S-adenosylmethionine
SULT	Sulfotransferase
TIC	Total ion current
tRNA	Transfer RNA
T_{max}	Time to reach C_{max}
UDPGA	UGP-glucuronic acid
UGT	UDP-glucuronosyltransferase
V	Volts
V_{max}	Maximum rate achieved by the system

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Chapter 1 – Introduction

1.1 - Sources, dietary abundance and intake of flavonoids

Flavonoids are a group of polyphenolic compounds widely distributed in nature, mainly in plant foods, and contain two or more aromatic rings (Heim et al., 2002, Harborne and Williams, 2000). These compounds have roles in flavour, pigmentation and plant physiology, and have been of great interest for the last two decades for their potential health benefits owing to their radical oxygen species (ROS) scavenging abilities (Iwashina, 2000). There is a lack of uniform distribution in the amount of flavonoids present in different plants; for example soy flour can contain 800 - 1800 mg/kg isoflavones, whereas tomatoes can contain as little as 2 – 15 mg/kg flavonols (Hertog et al., 1992, Franke et al., 1999, Coward et al., 1993). The richest sources of flavonols are onions (can be up to 1.2 g/kg), with considerable amounts also found in broccoli, blueberries, leeks, kale, tea, and red wine (Justesen et al., 1998, Herrmann, 1976, Hertog et al., 1993b, Crozier et al., 1997). These flavonols are generally present in the diet in their glycoside forms, with rhamnose or glucose being the most common sugar moiety (Veitch and Grayer, 2008). Polyphenol intake amongst the general population can reach up to 1 g/day in individuals who consume many portions of fruit and vegetables and varies dramatically based on the types of flavonoids and the populations studied (Erlund et al., 2002, Scalbert and Williamson, 2000). In Denmark, Holland, and the United States, flavonol consumption has been estimated at between 20 – 25 mg/day (Sampson et al., 2002, Justesen et al., 1997, Hertog et al., 1993a). Anthocyanins are consumed on average at 82 mg/day in populations who consume high amounts of berries and could exceed 200 mg/day (Mursu et al., 2008).

1.2 - Bioavailability of Flavonoids

Bioavailability has been defined by the US food and drug administration as "the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action" (Food and Drug Administration. Code of federal regulations, 2012). Polyphenols that are present in the diet at higher amounts do not necessarily have better bioavailability then polyphenols present in lower amounts; this is due to bioavailability-affecting factors, which include intestinal transit time, food matrix, colonic and phase II metabolism, chemical structure of the flavonoid, genetics, interactions with other compounds, absorption, and methods of consumption (Williamson and Manach, 2005, Manach et al., 2004, Schramm et al., 2003, Roura et al., 2007, Passamonti et al., 2002, Azuma et al., 2002).

Flavonoids undergo extensive UDP-glucuronosyl transferase, catechol-O-methyl transferase, and sulfotransferase mediated phase II metabolism before being passed into the circulation; forming methylated, sulfated, or glucuronide metabolites. The ATP-binding cassette (ABC) transporter family, which includes the P-glycoproteins (P-gp) and the multidrug resistance proteins (MRP), mediate the ATP dependent efflux of flavonoid metabolites back into the lumen of the intestine (Walle et al., 1999, Walgren et al., 2000, Hu et al., 2003). These factors taken together can severely limit the bioavailability of flavonoids and explain their generally low bioavailability.

1.3 - Bioactivity of flavonoids and their metabolites

Flavonoids are known to be extensively conjugated upon ingestion, Therefore, the bioactivity of flavonoids can at least in part, be attributed to their circulating metabolites. This applies to the anthocyanins. After ingestion, 60 – 90 % of Anthocyanins are rapidly degraded or metabolised, with approximately 72 % accounted for by the major metabolite; protocatechuic acid (Galvano et al., 2008). Upon metabolism, the structure of the compound is changed and the bioactivity may be altered. Generally

the bioactivity is thought to be reduced as metabolism typically inactivates ingested compounds and effectively marks them for excretion via the urine or bile (Tukey and Strassburg, 2000, Lu et al., 2003a). These metabolites can also be just as biologically potent as the parent compounds or sometimes even more biologically active, as is the case with morphine glucuronides (Paul et al., 1989). The effect of bioactivity can also depend on the site of conjugation. EGCG glucuronides at the 3' and 3'' positions had the same radical scavenging capability as EGCG, whereas the glucuronides at positions 7 and 4'' were less bioactive when comparing radical scavenging capacity (Lu et al., 2003). Most studies show that flavonoid metabolites do have lower bioactivities than their parent compounds (Delgado et al., 2014, Lambert et al., 2007). Most studies use deconjugated or parent compounds in bioactivity studies, not taking into account that many of the bioactivities could be due the metabolites. However, recently this has received more attention, owing to the realisation that most circulating forms of flavonoids in the plasma are conjugated (Renouf et al., 2011, Pietta et al., 1998, Meng et al., 2001, Mullen et al., 2004, Clifford et al., 2013).

1.4 - Summary

Polyphenols are consumed in high amounts by individuals who have high plant-based diets. A significant number of studies have been carried out over the last two decades that have correlated health effects with the intake of dietary polyphenols. These studies have also elucidated the metabolism and bioavailability of these flavonoids to a reasonable extent, which can further be interpreted as contributing to the benefits on human health (Williamson and Manach, 2005, Scalbert and Williamson, 2000). Although some mechanisms of flavonoids acting on cells have been better understood recently, much more rigorous and detailed studies are needed to find exact mechanisms of action and cellular targets. Most studies on mechanisms of action are carried out *in vitro*, using concentrations that are unachievable in the circulation and parent compounds which do not necessarily reflect the conjugated forms found in plasma, so it's imperative

to interpret these results carefully. The polyphenols should also be able to reach the target tissues/organs which are being studied and correlated with potential beneficial health effects. For example there is no point studying the effects of certain polyphenols on neurons *in vitro* if that particular flavonoid cannot cross the blood-brain barrier *in vivo*. Exceptions of course would be the study of polyphenols on intestinal cells, as they are directly in contact with ingested flavonoids in the GI tract. In studies of polyphenol health effects, especially in epidemiological studies, data have proven inconclusive regarding health effects in humans, and therefore are insufficient to claim outright positive effects of polyphenol consumption. Further research is required for the exact ADME properties of flavonoids to help elucidate exactly which metabolites contribute to certain health effects.

1.5 - Structure of thesis and aims

The thesis is organised into 7 chapters; a small general introduction to polyphenols and flavonoids, literature review, four chapters on experimental work, and a general discussion and conclusions chapter. There are currently no data in the literature regarding the absorption or uptake of green tea catechins in the oral mucosa and if there are any metabolites produced after absorption in the oral cavity. The aim of work presented in chapter 3 was to elucidate the absorption of green tea catechins in human oral cells, potential formation of metabolites, and the protective effects of those catechins against H_2O_2 induced mitochondrial DNA damage in the oral cavity.

Extensive studies on the metabolism of catechins have been carried out previously (Zhu et al., 2000, Vaidyanathan and Walle, 2002, Vaidyanathan and Walle, 2001, Spencer, 2003, Okushio et al., 1999, Lu et al., 2003b, Lu et al., 2003a). However what is lacking in these studies is data on the formation of catechin metabolites at regular time-points over a physiological time range which reflects the average length of transit through the GI tract in humans. Therefore the aim in chapter 4 was to analyse the metabolism of green tea catechins in different enzyme

fractions of intestine and liver, and catechol-O-methyl transferase from porcine liver. As the formation of metabolites of green tea catechins over 3 hours has not been previously reported, it was analysed at various time-points from 0 to 180 minutes.

Mitochondria are the main site of reactive oxygen species (ROS) production in the cell. The close proximity of mitochondrial DNA to these may make it more susceptible to ROS induced damage (Alexeyev, 2009). In chapter 5, it is hypothesised that green tea catechins and their metabolites (purified metabolites, and metabolite mixtures formed from enzyme assays in chapter 4) would protect mitochondrial DNA from damage in intestinal cell lines.

As the bioavailability of catechins is very low, chapter 6 looks at strategies to decrease metabolism of catechins *in vitro* for the potential application to *in vivo* studies to help increase bioavailability. Two dietary inhibitors of intestinal glucuronidation (piperine and quercetin) have been identified (Lambert et al., 2004, Mohamed and Frye, 2010) and it was hypothesised that catechin glucuronidation by intestinal microsomes would be decreased in the presence of these compounds. Chapter 7 contains general discussion and conclusions, and possible future directions.

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Chapter 2 - Literature review: green tea catechins

After water, tea is the most largely consumed beverage worldwide, estimated at 120 ml/day per capita consumption. Its popularity is attributed to its flavour, proposed health benefits, and its characteristic aroma. From the leaves of the plant *Camellia sinensis* there are approximately 300 different types of tea manufactured using various processing methods. However, there are three major commercial varieties of tea available: green (20% consumption), oolong (2% consumption), and black tea (78% consumption). Black tea is largely consumed in Europe and the USA, whereas in Asia green tea is most commonly consumed (Balentine et al., 1997, Katiyar and Mukhtar, 1996).

The various forms of tea are prepared from an infusion of plant leaves from the plant *Camellia sinensis*, which is today produced in over 30 countries worldwide, and for thousands of years has been cultivated in Asia. Each year approximately 2.5 million metric tonnes of tea are produced, which go through different processes of enzymatic oxidation or fermentation to make black tea, green tea, or oolong tea. These different processes give rise to varying chemical compositions (Matthew E. Harbowy, 1997).

Tea leaves are fully fermented to produce black tea, where the enzyme polyphenol oxidase (Kumar et al.) found in the plant, oxidises catechins present in the tea leaves to theaflavins and thearubigins. Green tea production involves the inactivation of the PPO enzyme and is prepared by steaming and drying the tea leaves, thus maintaining the catechins present in the tea leaves. Oolong tea undergoes partial fermentation, and ends up with chemical composition that is in between that of black and green tea (Balentine et al., 1997, Matthew E. Harbowy, 1997).

Green tea has been extensively studied for potential health beneficial effects, including prevention of cancer, heart disease, diabetes, and neurodegenerative diseases (Lambert et al., 2005, Yang et al., 2006, Higdon and Frei, 2003, Weisburger, 1999). The health benefits of tea are generally ascribed to its polyphenol content; the major polyphenols in green tea being catechins which are categorised by a di- or tri-hydroxyl

group substitution of the B ring and the meta-5,7-dihydroxyl substitution of the A ring (figure 1).

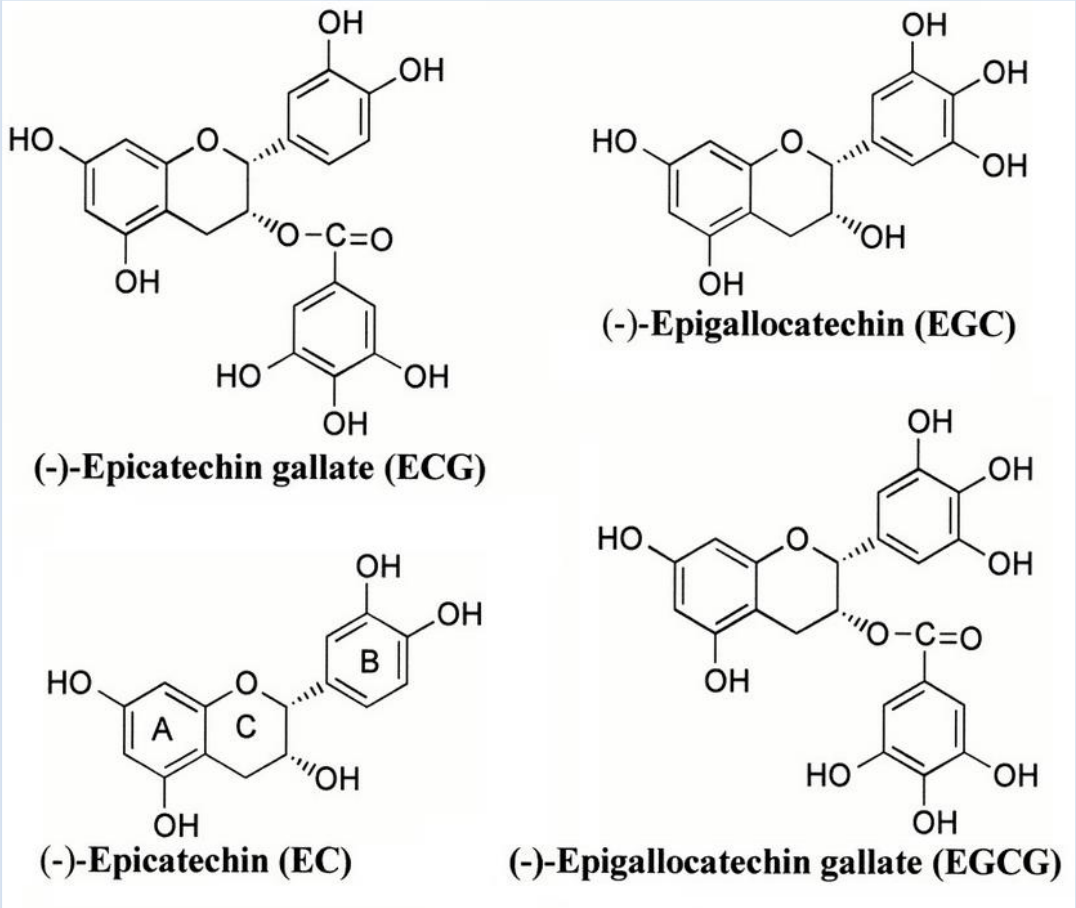

Figure 1 - Chemical structures of the four major Catechins

The average cup of brewed green tea has 30-40% catechins (dry weight), including: catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epigallocatechin-3-gallate (EGCG) (Balentine et al., 1997). EGCG is the most abundant (50-80% of total catechin content) and widely studied (Balentine et al., 1997, Graham, 1992).Green tea catechins have been shown to have potential beneficial health effects in studies of Parkinson's disease (Choi et al., 2002), Alzheimer's disease (Obregon et al., 2006, Ramassamy, 2006), stroke (Choi et al., 2004, Koh et al., 2006), obesity (Lin and Lin-Shiau, 2006), diabetes (Anderson and Polansky, 2002), and chemoprevention (Adhami et al., 2007, Clark and You, 2006, Kumar et al., 2007). Their high antioxidant activity makes them very efficient scavengers of peroxynitrite,

singlet oxygen, superoxide, and nitric oxide (Guo et al., 1999, Lambert and Elias, 2010). They also have potential as anti-inflammatory agents (Ahmed et al., 2006, Tedeschi et al., 2002), iron & copper ion chelators (Wang et al., 2007), antioxidant and other detoxifying enzyme upregulators (Khan et al., 1992, Chou et al., 2000), and in protection of DNA against oxidative damage (Luo et al., 2006).

Although catechins have long been characterized as powerful antioxidants *in vitro*, evidence suggests that these compounds undergo significant metabolism in the liver, small intestine, and colon. Biotransformation of catechins leads to the formation of glucuronide and sulfate conjugates, and methylated metabolites. Significant biotransformation of green tea catechins occurs in the colon where the resident microflora degrades them to small phenolic acids, some of which may be absorbed (Lu et al., 2003b, Vaidyanathan and Walle, 2002, Crespy et al., 2004, Lu et al., 2003a, Feng, 2006, Spencer, 2003, Zhu et al., 2000). It has been realised that very low bioavailability and extensive metabolism of green tea polyphenols in humans and animals reduces biological activity substantially (Manach et al., 2005, Williamson and Manach, 2005, Hollman et al., 1997).

2.1 Bioavailability of Catechins

Biological properties of polyphenols depend on their bioavailability. After ingestion, the metabolism of polyphenols should be carefully studied, since it is obvious that polyphenols should be bioavailable in some form or other to exert biological effects. There have been significant advances in our knowledge of polyphenol absorption and metabolism in the past few years, and it is evident that some classes of polyphenols (Isoflavones among others) are sufficiently absorbed to have the potential to exert biological effects (Hollman et al., 1997). One of the main objectives of bioavailability studies is to determine, among the hundreds of dietary polyphenols, which are better absorbed and which lead to the formation of active metabolites (D'Archivio et al., 2010). In most cases this is not done in a comparative way, as bioactivity depends on more than just bioavailability. There are

countless studies on the effects of polyphenols on biological systems *in vitro,* such as intestinal and hepatic cancer cell lines, but many of them have not taken bioavailability and metabolism factors into consideration, and the effects reported in these studies do not necessarily occur *in vivo*. Although most polyphenols are absorbed to some extent, this is usually also dependent on the type of polyphenol (Manach et al., 2005).

The range of concentrations required for an effect *in vitro* varies from 0.1 µM – 100 µM. However, physiological plasma concentrations do not exceed 10 µM (high end of plasma concentration range), hence the effects of polyphenols *in vitro* at concentrations of >10 µmol/L are generally not representative of the situation occurring *in vivo (Manach et al., 2005, Kidd, 2009, D'Archivio et al., 2010)*. In the case of catechins plasma concentrations rarely exceed 1 µM, and bioavailability studies of catechins are not usually consistent in their findings (Yang et al., 1999, Wiseman et al., 2001, Williamson and Manach, 2005, Lee et al., 2002, Henning et al., 2004). Furthermore, absorption is accompanied by extensive conjugation and metabolism, and the forms appearing in the plasma are usually different from the forms found in the food/beverage; e.g. the compound EGC in the beverage, is found mainly in the plasma as glucuronide and sulphate conjugates (Lee et al., 1995). This can lead to confounded attempts to compare *in vitro* findings with *in vivo* results in humans and animal models.

Some studies have investigated the kinetics and extent of polyphenol absorption and/or metabolism by measuring plasma concentrations and urinary excretion in humans after the ingestion of a single dose of polyphenol, provided as a pure compound, plant extract, or whole beverage (Hollman et al., 1997, Pietta et al., 1998, Harada et al., 1999, Chan et al., 2007, Hong et al., 2003, Hu et al., 2003, Renouf et al., 2011, Hong et al., 2002, Stalmach et al., 2009).

The daily consumption of catechins is estimated to be approximately 18–50 mg/d, with the main sources being tea, chocolate, apples, pears, grapes, and red wine. Although they are present in many foods and beverages, the bioavailability of catechins has been studied mainly after

the intake of green tea and cocoa (Arts et al., 2000, Williamson and Manach, 2005). Among catechins, bioavailability varies distinctly (Table 1). The bioavailability of green tea catechins is mainly determined via the analysis of biological fluids such as plasma and urine at various time-points after the consumption of green tea, catechins in a capsule, or green tea extract form (Del Rio et al., 2010, Henning et al., 2004, Stalmach et al., 2009). It is virtually impossible to accurately determine bioavailability of green tea catechins in humans, as ideally the bioavailability would be measured by comparison of the plasma concentrations of the non-conjugated catechins after oral and IV administration of the same dose. Also it would be helpful to analyse the concentrations of catechins and their metabolites in certain target organs, but due to the difficulty in accessing the organs in humans, studies on animals are conducted. The disadvantage to this is that the metabolism and bioavailability *may* vary significantly between animals and humans, further adding difficulty to accurate determinations of bioavailability in humans.

As already indicated, the favourable health effects of tea catechins are essentially associated to their bioavailability, absorption, distribution, metabolism, and excretion from the body (Figure 2). Catechins are quite high molecular weight compounds (300-450 Da) consisting of more than 5 hydroxyl groups, and according to Lipinski's rule of five, they should have low bioavailability due to their large size (Lipinski et al., 2001). When consumed in representative amounts of 1-2 cups/100-200 mg of catechins, the level of catechins and other flavonoids in plasma does not exceed 1 μM. The overall plasma concentration of catechins (conjugated and free) is approximately 2-3 μM, or less (Yang et al., 1998), (Warden et al., 2001).

Approximately 1-2 hours after tea consumption, the concentration of catechins in human plasma increases. However, the bioavailability of catechins is rather low — only 0.2–2 % of the consumed amount of catechins gets into plasma of healthy humans, in parent and conjugate forms (Wiseman et al., 2001).

The bioavailability of pure EGC, ECG, and EGCG in healthy volunteers was investigated. After consumption of 1.5 mM EGC, ECG, and EGCG

alone, the mean plasma concentration was 5 μM, 3.1 μM, and 1.3 μM respectively (van het Hof et al., 1998). These plasma concentrations are at the upper limit of concentrations obtained, and are not achievable by drinking green tea without supplementation with pure catechins.

After oral consumption of 100 to 1600 mg of pure EGCG, 0.26 to 6.35 μM of EGCG was detected in plasma in 2-3 hours; some metabolites of EGCG at concentrations of 0.28 to 7.40 μM were also found between 1.9 - 4.6 hours (Ullmann et al., 2003). Again, these high plasma concentrations are only found after ingestion of very high doses of pure catechins, and cannot be achieved after consumption of reasonable amounts of green tea alone. To ingest up to 1600 mg of EGCG from green tea, one would have to drink between 8 – 16 cups per day.

Del Rio *et al* studied the bioavailability of GTCs after the consumption of tea, and the urine of volunteers was analysed over 24 hours (Del Rio et al., 2010). Eight catechin metabolites and unconjugated gallic acid were identified in urine. Urine contained 7.2% of GTCs and 4.5% of gallic acid in relation to their total amounts contained in the consumed tea. The C_{max} of 5 metabolites (methyl-epigallocatechin glucuronide, epigallocatechin glucuronide, methyl-epicatechin sulfate, epicatechin sulfate, and epicatechin glucuronide) were observed in the urine 4 hours after consumption of the tea; the C_{max} level of 2 metabolites (methyl-epigallocatechin sulfate and methyl-epigallocatechin) were present in the urine after 10 hours; whereas the content of epigallocatechin in the urine was seen as continuously increasing over the period of 24 hours. A previous study by Stalmach *et al* showed similar bioavailability to this study (Stalmach et al., 2009). Absorption, metabolism, and excretion in urine of GTCs were studied by HPLC-MS in 10 healthy volunteers who were taking 500 ml of Choladi green tea, containing 648 μM of flavan-3-ols (Stalmach et al., 2009). After consumption of green tea, plasma and urine were analysed over 24 hours. Ten methylated, sulfated, and glucuronide metabolites of EC and EGC at a concentration of 29-126 nM were found to occur 1.6-2.3 hours after consumption. This indicates that absorption occurs in the small intestine. Plasma also contained unconjugated EGCG and ECG at concentrations of 55 and 25 nM, respectively. Fifteen

metabolites of EC and EGC were identified in urine. No metabolites of EGCG and ECG were detected. Overall green tea catechin excretion was estimated at 8.1% of intake but excretion of certain flavanols varied from 11.4% to 28.5% of intake. These studies show that epicatechins are more bioavailable than other catechins in green tea.

The data shows that bioavailability of the different catechins varies significantly in different studies. It is estimated that at any one time, 80% of green tea catechins are in the urine and plasma in their conjugated forms, which can still scavenge superoxide free radicals, as some metabolites retain their intact hydroxyl substituents (Kuhnle et al., 2000, Vaidyanathan and Walle, 2002, Li et al., 2001, Li et al., 2000). The doses of GTCs, populations, and matrices of delivery used in bioavailability studies are usually not kept constant, which could give one explanation for the inconsistencies in the data obtained in different bioavailability studies. Another inconsistency is the differences in methodologies used to quantify and analyse GTCs in biological fluids.

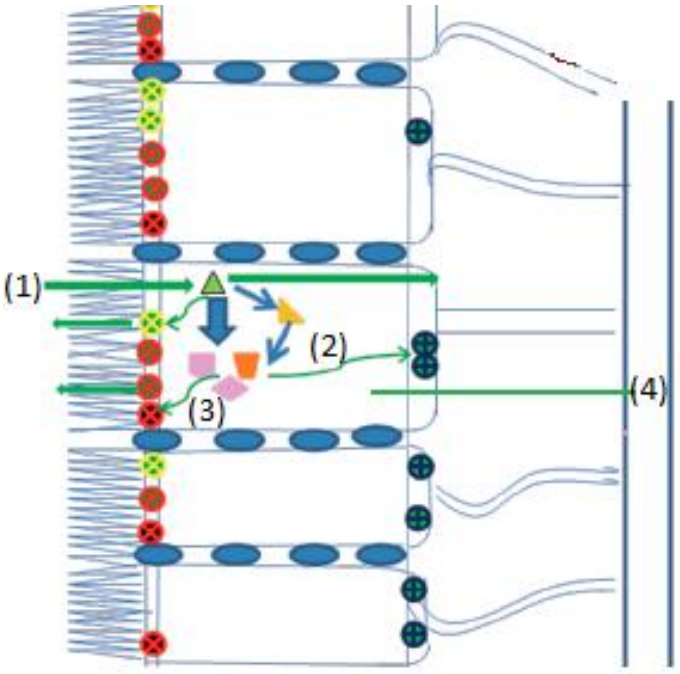

Figure 2. The pathways taken by catechins through the enterocyte. Absorption (1), intracellular metabolism (2), conjugation (3), and efflux across membrane (4).

Source	Dose (mg)	T_{max} *(h)*	Plasma conc *µmol/L*	Half-life *(h)*	Reference
Pure EGCG	50-1600	1.3–2.2	0.28- 7.4	1.9–4.6	(Ullmann et al., 2003)
	200-800	1.8–4	0.16-0.96	1.9–3.1	(Chow et al., 2001)
Polyphenon E	200-800	2.4–4.1	0.16-0.82 (EGCG)	1.9–3	(Chow et al., 2001)
	164 (TC's)	-	0.56 TC's at 3 h	-	(van het Hof et al., 1998)
Green tea powder	105 EGCG	2.0	0.14–0.31 EGCG	-	(Unno et al., 1996)
Pure ECG	663	4.0	3.1 ECG	6.9	(Van Amelsvoort et al., 2001)
Green tea extract	225-525 EGCG		0.66-4.4 EGCG at 1.5h		(Nakagawa et al., 1997)
	7.5-17.5 EGC		0.03-0.25 EGC at 1.5h	-	(Nakagawa et al., 1997)
	88 EGCG	-	0.24 EGCG at 1 h	-	(Yang et al., 1998)
	82 EGC	-	0.46 EGC at 1 h	2.0 TC's	(Yang et al., 1998)
	109.5-328 EGCG	1.6- 2.7	0.26-0.70 EGCG	4.9-5.5	(Kimura et al., 2002)
	102-306 EGC	1.3-1.8	0.48-1.8 EGC	2.5- 2.8	(Kimura et al., 2002)
	37.5-112.5 EC	1.4-1.8	0.19-0.65 EC	3.2-5.7	(Kimura et al., 2002)
Green tea	640 (TC's)	1.5	1.8 TC's	-	(van het Hof et al., 1999)
	1040 (TC's/d for 3days)	0.5–2.0	1.0 TC's	-	(Pietta et al., 1998)

Table 1- Non-exhaustive list of bioavailability studies of catechins. (TC's: total catechins)

2.2 Metabolism of Catechins

The major biotransformation reactions that GTCs undergo are methylation, glucuronidation, sulfation, and ring-fission metabolism (Li et al., 2001,

Kohri et al., 2001, Hu et al., 2003, Lu et al., 2003b, Lu et al., 2003a, Meng et al., 2002, Feng, 2006).

2.2.1 Methylation

Catechol-*O*-methyltransferase (COMT) is present in all tissues at high activity in humans and rodents, and catalyzes the metabolic O-methylation of various catecholic compounds (Axelrod and Tomchick, 1958). COMT has highest activity in liver, then kidney and gastrointestinal tract, and has been found in all mammalian tissues investigated which include human, mouse, and rat (Lu et al., 2003b).

COMT enzyme studies (Lu et al., 2003b, Okushio et al., 1999b, Okushio et al., 1999a) show that the catechins are readily methylated to form: 3'-O-methyl-catechin (Donovan et al., 1999), 4'-*O*-methyl-catechin (Donovan et al., 1999), 3'-*O*-methyl-EC (Okushio et al., 1999b, Okushio et al., 1999a), 4'-*O*-methyl-EC (Okushio et al., 1999b, Okushio et al., 1999a), 4'-*O*-methyl-EGC (Lu et al., 2003b), 4''-*O*-methyl-ECG (Okushio et al., 1999b, Okushio et al., 1999a), 4''-*O*-methyl-EGCG (Okushio et al., 1999b, Meng et al., 2002), and 4',4''-*O*-dimethyl-EGCG (Lu et al., 2003b) (figure 3).

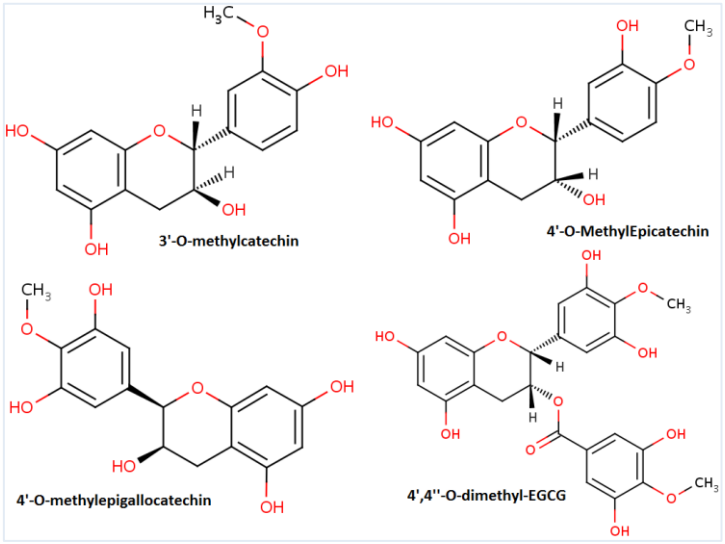

Figure 3 - Structures of some methylated catechins indicating position of methylation (phenol explorer.eu)

Epicatechin, and epigallocatechin were good substrates for metabolic *O*-methylation by placental cytosolic COMT (150–500 pmol/mg of protein/min), but epicatechin gallate and epigallocatechin gallate were *O*-

methylated at much lower rates (<50 pmol/mg of protein/min) (Zhu et al., 2000). *O*-methylation by human placental COMT showed dependence on incubation time, cytosolic protein concentration, incubation pH, and concentration of *S*-adenosyl-L-methionine (the methyl donor).
Rat liver cytosol shows higher COMT activity toward EGCG and EGC than human or mouse liver cytosol and the small intestine had lower specific activity than the liver in the methylation of EGCG and EGC.
Glucuronidation on the B-ring or the D-ring of EGCG greatly inhibited the methylation on the same ring, but glucuronidation on the A-ring of EGCG or EGC did not affect their methylation (Lu et al., 2003b).
Meng et.al chemically synthesized 4'-*O*-methyl EGC, 4''-*O*-methyl EGCG, and 4', 4''-di-*O*-methyl- EGCG and confirmed their structures in human plasma and urine samples using LC/MS and found that 4'-*O*-methyl EGC and 4', 4''-di-*O*-methyl-EGCG were the major methylated metabolites of EGC and EGCG in humans (Meng et al., 2001, Meng et al., 2002). At low concentrations of EGCG, the dimethylated compound is the major product, whereas at high EGCG concentrations, monomethylated EGCG metabolites increase (Zhu et al., 2000, Okushio et al., 1999b, Lu et al., 2003b).

2.2.2 Glucuronidation

UDP-glucuronosyltransferase (UGT)-catalyzed glucuronidation is a major pathway in Phase II metabolism. Catechins are conjugated to form the metabolites EGCG-7-Gluc, EGCG-4''-Gluc, EGCG-3''-Gluc, EGCG-3'-Gluc, EGC-3'-Gluc, and EGC-7-Gluc, from EGCG and EGC (Li et al., 2001, Lu et al., 2003a, Sang and Yang, 2008). Catechin 5-O-β-glucuronide and epicatechin 5-O-β- glucuronide were only found in rat, but have not been identified in human liver, human small intestine, and human colon microsomes (Harada et al., 1999, Vaidyanathan and Walle, 2002).

Crespy et al identified 3 peaks on a HPLC profile for ECG glucuronidated metabolites using rat intestinal and liver microsomes, but glucuronidation positions of ECG were not reported in the paper. They also showed rat

intestinal microsomes exhibited higher activity on the galloyl group of ECG and EGCG compared to the flavonoid ring, whereas hepatic glucuronidation was higher on the flavonoid ring of EGCG and ECG compared to the galloyl groups (Crespy et al., 2004).

Natsume et al purified and elucidated the chemical structure of glucuronidated epicatechin metabolites in human and rat urine after oral administration. Metabolites purified from human urine were epicatechin-3'-*O*-glucuronide, 4'-*O*-methyl-epicatechin-3'-*O*-glucuronide, and 4'-*O*-methyl-epicatechin-5-*O*-glucuronide or 4'-*O*-methyl-epicatechin-7-*O*-glucuronide (according to ^{1}H- and ^{13}C-NMR, HMBC, and LC-MS analyses). The metabolites purified from rat urine were 3'-*O*-methyl-epicatechin, epicatechin-7-*O*-glucuronide, and 3'-*O*-methyl-epicatechin-7-*O*-glucuronide. These compounds were also detected in the blood of humans and rats by LC-MS (Natsume et al., 2003). More recently, a new method identified EC-glucuronides in dogs (Mata-Bilbao Mde et al., 2007). EGCG-4''-Gluc was found to be the major metabolite formed by human, mouse, and rat microsomes (Lu et al., 2003a). Mouse small intestinal microsomes had the greatest catalytic efficiency for the formation of EGCG-4''-*O*-glucuronide followed by mouse liver, human liver, rat liver, and rat small intestine.

The UGT-catalyzed glucuronidation of EGC was much lower than that of EGCG. Again, mouse liver microsomes had the greatest catalytic efficiency for the formation of EGC-3'-*O*-glucuronide followed by human liver, rat liver, and rat and mouse small intestine (Lu et al., 2003a). Human UGT1A1, 1A8, and 1A9 had the highest glucuronidation activity toward EGCG (Lambert et al., 2007). In some instances Catechin and EC are completely conjugated in plasma, however, EGCG and ECG are notable exceptions amongst flavonoids and are present mainly as the unconjugated parent compound in plasma (Crespy et al., 2004).

2.2.3 Sulfation

The main sulfotransferase isoform in the human liver is SULT1A1 (Vaidyanathan and Walle, 2002). SULT1A1 and SULT1A3 have high expressions in the human small intestine (Sundaram et al., 1989,

Vaidyanathan and Walle, 2002). EGCG is time- and concentration dependently sulfated by human, mouse, and rat liver cytosol. The rat cytosol has the greatest activity to sulfate EGCG, followed by the mouse and the human liver cytosol. The sulfates of EC, EGC, and EGCG have been identified from rodent and human samples mainly through LC/MS analysis. However, the chemical structures of those sulphate metabolites have not been characterized. The active sites for sulfation of tea catechins are still unknown (Sang et al., 2011).

In human urine samples, EGC-*O*-Sulfate and EC-*O*-Sulfate were the major sulfated metabolites formed from EGC and EC respectively (Li et al., 2001). Recent results from mouse urine samples analysed using tandem mass spectrometry showed that methylated EGCG (or glucuronidated or sulfated EGCG) can be further glucuronided and/or sulfated (or methylated) to form the mixed EGCG metabolites *O*-Methyl-EGC-*O*-Sulfate, *O*-Methyl-epicatechin-*O*-Sulfate, M4-*O*- Sulfate, and M6-*O*-Sulfate (Lambert et al., 2007).

2.2.4 Glucosidation and Thiol conjugation

Sang and Yang identified a novel EGCG metabolite, 7-*O*-*β*-D-glucopyranosyl-EGCG-4''-*O*-*β*-D-glucupyranoside, in a mouse urine sample using liquid chromatography /electrospray ionization tandem mass spectrometry. The structure of this metabolite was confirmed by analyzing the MS^n (n = 1–4) spectra as well as comparing the MS/MS spectra of its product ions with those from EGCG and EGCG-4″-*O*-*β*-D-glucupyranoside standards. To their knowledge, this was the first report of the identification of a glucoside metabolite of EGCG in mammals. The results indicated that glucosidation represents a novel pathway in the metabolism of EGCG in mice (Sang and Yang, 2008).

Catechin can be metabolised by tyrosinase to form a cytotoxic *o*-quinone, which reacts with glutathione to form mono-, bi-, and triglutathione conjugates of catechin and mono- and biglutathione conjugates of a catechin dimer (Moridani et al., 2001). When peroxidase and hydrogen peroxide were used, only monoglutathione conjugates of catechin were

formed. In the presence of NADPH, rat liver microsomes also catalyzed oxidation of catechin leading to glutathione conjugate formation.
Sang et al found that EGCG can be oxidized by peroxidase and hydrogen peroxide and then reacted with cysteine or glutathione to form conjugates. The structures of the cysteine and glutathione conjugates of EGCG were identified using 2D NMR and MS. Two thiol conjugates of EGCG (2'-cysteinyl EGCG and 2''-cysteinyl EGCG) were identified by LC/ESI-MS^2 analysis from the urine samples of mice administered 200 or 400 mg/kg EGCG, i.p. These conjugates were not found in urine samples of mice after receiving EGCG at 50 mg/kg i.p. or in human urine following consumption of 3 g of decaffeinated green tea solids (containing 333 mg EGCG). They concluded that EGCG is believed to be oxidized to form EGCG quinone, which can react with glutathione or cysteine to form the thiol conjugates and that detectable amounts of thiol conjugates of EGCG are formed only after rather high doses of EGCG are given to the mice (Sang et al., 2005, Sang et al., 2011). It would be worthwhile to further study whether glucosidation of EGCG occurs in humans. Further studies are needed to determine whether glucosidation is similar to glucuronidation as a general biotransformation pathway to most of the dietary flavonoids.

2.2.5 Microbial metabolites in colonic flora

There is a distinctive flora present in the different portions of the gastrointestinal tract i.e. mouth, oesophagus, stomach, small intestine, and large intestine. The human colon contains microbes numbering in the trillions, coming from approximately 500 different species of bacteria, which have enormous hydrolytic and catalytic potential (Backhed et al., 2005, Savage, 1977, Salminen et al., 1996).
The colonic flora breaks down polyphenols to constituent compounds such as phenolic acids and their glycine conjugates. Bacterial enzymes catalyse many reactions including hydrolysis, hydrogenation, dehydroxylation, oxidation, ring cleavage, decarboxylation and rapid de-conjugation.
Studies have demonstrated that uptake/absorption of GTCs in the small

intestine is relatively small. The low absorption in the small intestine implies that the majority of ingested GTCs including those absorbed and metabolised will reach the colon where they come across the colonic microflora. It has been shown that colonic metabolism of tea catechins form the ring fission products 5-(3',5'-dihydroxyphenyl)-γ-valerolactone (M6'), 5-(3',4',5'-trihydroxyphenyl)- γ -valerolactone (M4), and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone (M6) (Li et al., 2001, Li et al., 2000). Maximal urine concentrations of 8, 4, and 8 μM were shown for M4, M6, and M6', respectively following ingestion of 200 mg EGCG. Anaerobic fermentation of EGC, EC, and ECG with human fecal microflora resulted in the production of M4, M6, and M6' (Meselhy et al., 1997). Further degradation of these compounds by intestinal flora results in the formation of lower molecular weight phenolic acids. Urine samples collected at 6-48h contained detectable amounts of final catechin metabolites, including 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-methoxy-4-hydroxy-hippuric acid and 3-methoxy-4-hydroxybenzoic acid (vanillic acid) (Pietta et al., 1998).

2.2.6 Active Efflux (Phase III metabolism)

The multidrug resistance-associated proteins (MRP) are ATP-dependent efflux transporters that are expressed in many tissues and over expressed in many human cancers (Lambert et al., 2007, Leslie et al., 2001). MRP1 is located on the basolateral side of cells and is present in nearly all tissues and transports compounds from the interior of the cells into the interstitial space (Leslie et al., 2001). MRP2 is located on the apical surface of the intestine, kidney, and liver, where it transports compounds from the bloodstream into the lumen, urine, and bile, respectively (Leslie et al., 2001). This process limits the bioavailability and cellular accumulation of many compounds, including green tea catechins.

Recent studies on EGCG uptake in Madin-Darby canine kidney (MDCKII) cells showed that indomethacin (MRP inhibitor) increased the intracellular accumulation of EGCG, EGCG 4''- *O*-methyl-EGCG, or 4',4''-di-*O*-methyl-EGCG by 10-, 11-, or 3-fold overexpressing MRP1 (Hong et al., 2003).

Similarly, treatment of MRP2 overexpressing MDCKII cells with MK-571 (an MRP2 inhibitor) resulted in 10-, 15-, or 12-fold increase in the intracellular levels of EGCG, 4''-*O*-methyl-EGCG, and 4', 4''-di-*O*-methyl-EGCG, respectively. Treatment of PGP overexpressing MDCKII cells with PGP (P-glycoprotein) inhibitors, however, resulted in no significant effect on the intracellular levels of EGCG or its metabolites. Treatment of HT-29 human colon cancer cells with indomethacin resulted in increased intracellular accumulation of EGCG and its methylated and glucuronidated metabolites (Hong et al., 2002). These data suggest a role for MRPs, but not PGP, in affecting the bioavailability of EGCG and its metabolites. Other studies showed that treatment of Caco-2 cells with MK-571 enhances apical to basolateral movement of EC and ECG (Vaidyanathan and Walle, 2001, Vaidyanathan and Walle, 2003). MK-571 reduced the efflux of EC-sulfates from the cytosol to the apical well, suggesting the EC-sulfates are also substrates for MRP2 (Vaidyanathan and Walle, 2001). Future studies should focus on the efflux transporters to determine if they efflux intact polyphenols preferentially compared to their metabolites, or vice versa. Lambert et al have suggested that the uptake of EGCG into HT-29 cells is mainly through passive diffusion (Lambert et al., 2007), others have demonstrated that ECG is a substrate for the monocarboxylate transporter (MCT). Inhibition of MCT-1 by benzoic acid or phloretin significantly reduced ECG uptake by Caco-2 cells (Lambert et al., 2007, Vaidyanathan and Walle, 2003).

The combined effects of MRP1, MRP2, and MCT on the bioavailability of the tea polyphenols remain to be determined *in vivo*. The apical location of MRP2 suggests that it acts to limit EGCG bioavailability by actively exporting EGCG back into the intestinal lumen. The remaining fraction of EGCG would then be absorbed into the portal circulation, enter the liver, and could subsequently be effluxed by MRP2 located on the canalicular membrane of the hepatocytes. In contrast, MRP1 is located on the basolateral side of enterocytes, hepatocytes, and other tissues. Substrates of this pump are effluxed from the interior of the cells into the intestinal space. The role of MRP1 would be expected to increase the bioavailability of EGCG *in vivo*. The influence of MRP1 and 2 *in vivo*, however, is likely to

depend on their relative tissue distribution. It was reported that the MRP2 mRNA level was over 10-fold higher than that of MRP1 in the human jejunum; therefore, efflux of EGCG by MRP2 may be predominant in the intestine, resulting in a decrease of EGCG bioavailability (Vaidyanathan and Walle, 2003, Vaidyanathan and Walle, 2001, Lambert et al., 2007, Taipalensuu et al., 2001).

A more recent study investigated the kinetics of efflux transport of the four major catechins in Caco-2 cell lines, to provide comparison on the efflux transport between each catechin. The basal-to-apical transport of each GTC at concentrations ranging from 15 to 265 μM was examined using the Caco-2 cell monolayer model. Transported amount of GTC was measured by high performance liquid chromatography with electrochemical detection. Kinetic parameters, V_{max}, K_m and Vmax/Km were determined and compared among the four studied GTCs. The extent of basal-to apical transport was, in descending order, EC > EGC > ECG ≈EGCG (Chan et al., 2007). Future studies should focus on the bioavailablity of tea polyphenols and specifically their metabolites *in vivo* and the effects of MRPs 1 and 2 on *in vivo* bioavailability.

2.3 Conclusions

Green tea - along with its constituent catechins- is considered as an important component in nutrition to prevent lifestyle-related diseases especially cancer and other denegenerative diseases. The health benefits of green tea are often attributed to polyphenols especially EGCG, recognized as an effective chemo-preventive agent that holds the ability to scavenge free radicals and is certainly helpful against various cancers..

There are currently strategies such as phytosome complexes that have enough data to claim that essentially they can double the bioavailability of certain polyphenols such as EGCG (Kidd, 2009). This could improve efficacy without compromising on safety issues as the product is natural and not chemically modified or synthesised.

Future studies should focus on improving the bioavailability and biological activity of GTCs in vivo, improving the absorption and metabolism, hence improving the health benefits obtained from drinking green tea. A direct comparison should only be made between studies on bioavailability when methods, matrices, populations, and doses are kept constant, which would decrease the discrepancies between published data. If the studies are to reflect how people generally consume tea, then instead of one dose followed by immediate analysis of plasma or urine, another avenue to explore would be repeat dosing which would represent a normal person drinking a cup of tea every ~4hours. As the general view is that green tea has more beneficial effects on health, most of the studies are understandably on green tea. However, in the west black tea is much more widely consumed, so it would seem sensible to also pay more attention to bioavailability studies on black tea.

It is also clear that in the case of catechins except EGCG, the catechins are usually found in plasma in the conjugated form, so more research needs to be done on the bioactivities of the metabolites circulating in the plasma. What would also be essential to include in further studies is the potential importance of colonic metabolites which are present in high concentrations in the urine

2.4 References

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Chapter 3 – Absorption of green tea catechins in the oral cavity, and protection against Hydrogen peroxide-induced mitochondrial DNA damage

Abstract

Background: Green tea catechins have been shown to have poor absorption, extensive metabolism, and low bioavailability in humans. The oral cavity is constantly being challenged by ingested or environmental factors, which can have negative effects on the cells mitochondria. It was hypothesised that green tea catechins at concentrations achievable in the oral cavity could be absorbed into human oral cells and protect against H_2O_2 induced mitochondrial DNA damage. **Methods:** Human volunteer buccal mucosal cells and H400 cells were used to measure absorption of catechins. Volunteers incubated green tea in the mouth for up to 15 minutes, whereas in H400 cells, catechins were incubated for up to 60 minutes. For mtDNA damage analysis, physiological concentrations of catechins were added to H400 cells before being challenged by H_2O_2. **Results:** ROS levels in buccal mucosal cells were significantly reduced when green tea was incubated in the mouth for up to 15 minutes. In human buccal cells, only EGCG and ECG were absorbed, whereas in H400 cells, all four catechins EGCG, EGC, ECG, and EC were significantly absorbed over 60 minutes. Physiological concentrations of catechins significantly protected H400 cells against H_2O_2 induced mtDNA damage; at 3 hours between 72.3 – 93.9 % (10 µM), and 35.4-88.5 % (2 µM). When incubating with catechins for 1 hour the protective effects were 46.2-78.7 % (10 µM). The gallated catechins showed a greater absorption in H400 cells than the non-gallated catechins. **Conclusions:** catechin incubation with oral H400 cells resulted in significant absorption of catechins, and afforded significant protection against mtDNA damage. These data show a potential beneficial effect of green tea catechins in the oral cavity.

3.1 – Introduction

The oral cavity consists of a specialised interface which includes the gingival epithelium (soft tissue) (Pollanen et al., 2003). Oral, sulcular, and junctional epithelium make up the gingival epithelium which is continuously challenged against ingested materials and microorganisms (Schroeder and Listgarten, 1997). The gingival epithelium protects the oral cavity and the connective tissue beneath it by acting as a physical barrier barring the invasion of any microorganisms; stimulating the entry of immune cells such as neutrophils via the secretion of chemokines and cytokines (Dale, 2002). It is also a source of antimicrobial products termed epithelial antimicrobial peptides (EAP), which have been shown to be effective against a varied range of microbes as well some viruses and yeast (Diamond et al., 2008, Schonwetter et al., 1995). Keratinised and non-keratinised layers make up the oral epithelium; the keratinised layer contains corneal, granular, spinous, and basal layers; and the non-keratinised layer includes superficial, intermediate, spinous, and basal layers. These oral epithelial layers consist of stratified squamous epithelium. In direct contact with the oral epithelial cells is the *lamina propria* (dense connective tissue) which contains *inter alia* fibroblasts, salivary glands, blood vessels, and structural fibers (Sloan et al., 1991, Squier and Kremer, 2001, Abdulmajeed et al., 2013, Richard and Pillai, 2010, Collins and Dawes, 1987).

The oral cavity undergoes a large range of stress from ingested compounds, and possibly toxins or microbes; as well as chemical and mechanical damage which can inhibit its ability to safeguard organs and tissues underneath (Squier and Kremer, 2001). In spite of the oral mucosa encountering these stressors, the epithelial tissue in the oral cavity is regenerated constantly via cell division. Thus damaged DNA is repaired before mitosis as this is when DNA is most susceptible to damage from external stressors (Thomson et al., 1999, Bjarnason et al., 1999). If after this constant exposure to risk factors and external stressors genomic integrity is compromised, through discrete genetic changes at the molecular level, oral cancers can develop especially after exposure to the

main risk factors such as alcohol consumption and smoking (Cruz et al., 2002). These factors account for approximately 60 % of oral cancers, whereas the remaining 40 % of cases are caused by unknown nutritional, environmental, genetic, or viral factors (Hashibe et al., 2009).

Tea is the second most widely consumed beverage after water and has been studied widely for its health benefits against degenerative diseases, cancer, heart disease, and diabetes (Butt and Sultan, 2009, Higdon and Frei, 2003, Lambert et al., 2005, Weisburger, 1999, Yang et al., 1999a, Yang et al., 2006). The polyphenolic catechins of green tea (EGCG, ECG, EC & EGC) in particular have been studied for their beneficial effects on human health as they comprise the majority of the dry weight compounds of green tea (Graham, 1992, Lambert and Elias, 2010, Sang et al., 2011).

Tea studies have also shown benefits towards prevention of dental caries, halitosis, stress-induced inflammation, and oral malignancies (Tsao et al., 2009, Yamamoto et al., 2003, Srinivasan et al., 2008, Maruyama et al., 2011, Lodhia et al., 2008, Hirasawa et al., 2006, Zhang and Kashket, 1998, Ferrazzano et al., 2011, Magalhaes et al., 2009). Tea has been presented as a possible chemopreventive agent against oral squamous cell carcinoma (OSCC) *in vitro* by inhibiting angiogenesis (Masuda et al., 2002, Cao and Cao, 1999), apoptotic cell death (Hsu et al., 2002, Babich et al., 2005), and reducing cell growth (Cao and Cao, 1999, Khafif et al., 1998, Elattar and Virji, 2000). 7,12-dimethylbenz[a]anthracene (DMBA)-induced HBP carcinogenesis was also shown to be inhibited by tea, owing to possible inhibition of angiogenesis and induction of apoptosis (Li et al., 1999, Li et al., 2002). Green tea catechins can accumulate in saliva after consuming 2-3 cups of green tea; EGCG 5-22 µg/ml, EGC 12-44 µg/ml, EC 1.8-7.5 µg/ml, whereas EGC was not determined (Yang et al., 1999b), and after chewing 2 g of green tea leaves for 5 minutes EC 6-28 µg/ml, EGC 14-160 µg/ml, EGCG 31-90 µg/ml, and ECG 11-28 µg/ml. Thus a total salivary catechins concentration of 12-260 µM can be achieved, which was shown to be able to inhibit oral cavity carcinoma cell proliferation (Lee et al., 2004).

Aims of study

Oxidative stress from reactive oxygen species (ROS) was first proposed by Harman *et al* as a major factor in ageing via damage to nucleic acids and cellular components (Harman, 1956). Since mitochondria are the target and production site of ROS (Harman, 1972), mitochondrial DNA is the target of oxidative ROS mediated damage, accumulating deletions and point mutations that can be transported and accrued in replicating cells (Miquel et al., 1983, Miquel et al., 1980, Fleming et al., 1982). Mitochondrial DNA (mtDNA) has a less proficient repair machinery in place compared to nuclear DNA, lacks protective histones, mutated molecules can be complemented by wild-type molecules (all of which help maintain the damage in cells), and has a mutation rate which is up to 15-fold higher, giving rise to the mitochondrial theory of ageing (Short et al., 2005). If these mutations are constant and begin to accumulate, they may reach sufficient levels to start causing damaging effects in the mitochondria, which can in turn lead to faster ROS production and mitochondrial dysfunction (Wallace, 2010). Dietary strategies for the prevention of excess ROS in the mitochondria may provide therapeutic effects in the oral cavity and other tissues where oxidative stress is prevalent. The aims of this study were therefore to investigate the absorption of green tea catechins and possible formation of metabolites in human buccal mucosal cells and immortalised H400 oral cell lines. It was hypothesised that exposure to catechins would protect oral cells against hydrogen peroxide induced mtDNA damage.

3.2 – Materials and methods

3.2.1 – Chemicals and reagents

Epigallocatechin-3-gallate, Epigallocatechin, Epicatchin-3-gallate, Epicatechin, Ascorbic acid, Hydrogen peroxide, Hydrocortisone Succinate, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), cell lysis buffer, and 4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate (Tiron) were purchased from Sigma Aldrich (Gillingham, UK). Green tea (Clipper fair trade pure green tea) for the

human study was purchased from the local supermarket. 2', 7'-Dichlorodihydrofluorescin-Diacetate (DCFH-DA) for the intracellular antioxidant assay was obtained from Cambridge Bioscience Ltd, Cambridge. The following were acquired from Life Technologies (Invitrogen): Dulbecco's modified Eagle's medium [DMEM:HAMS F12 (1:1)]; RPMI-1640 medium; foetal Bovine serum (FBS); Trypsin-EDTA; and L-Glutamine. HPLC-grade Acetonitrile; phosphoric acid; and methanol were purchased from Fisher scientific (Leicestershire, UK). UPLC-grade Acentonitrile; UPLC-grade methanol; and Formic acid were purchased from Biosolve BV (Valkenswaard, Netherlands). SYBR-green was obtained from Applied Biosystems (Life Technologies, Netherlands). Primers for Mitochondrial DNA fragments were purchased from Eurofins Genomics (Ebersberg, Germany). The Genomic DNA purification kit was purchased from ThermoScientific (Leicestershire, UK). The cell fractionation kit, consisting of the membrane and cytoplasmic isolation buffers; was obtained from cell signalling technology (New England Biolabs, Hertfordshire UK).

3.2.2 – Human intervention study

Nine volunteers were recruited for this small pilot study to determine the antioxidant capacity and absorption of green tea catechins in the oral mucosa. All participants were given detailed information about the experimental procedures and what was expected of them if they agreed to take part in the study. A Q&A visit was also scheduled so that the volunteers could ask questions and clear up any uncertainties they had. Volunteers were then signed an informed consent form before the commencement of the study. Volunteers were asked to avoid flavonoid-rich beverages and foods for 3 days prior to the study. Compliance was assessed via participant food diaries. Volunteers brushed the inside of their cheeks with their toothbrush the night before the experiment and did not have breakfast on the morning of the study days, each participant was sampled at every time point with each study day being assigned a different time point. Before drinking the green tea, participants brushed the inside of their left cheek and rinsed the cheek

with a 0.9% NaCl solution (baseline untreated cells). In this case these cells were used as control cells rather than using hot water as a control, as the participants did not consume any breakfast on the morning of the study days. This would have had minimal impact on the subsequent data obtained. Thereafter, freshly prepared green tea (one tea bag brewed in 450 mL boiling water) was held within the mouth over a period of 2.5 - 20 minutes and the buccal cells were collected from the right cheek (treated cells). Both sets of samples were washed three times with cold RPMI buffer, and then split into three equal aliquots, for the antioxidant assay and HPLC analysis of intracellular extracts.

3.2.3 – Antioxidant assay

Baseline, Trolox, and green tea treated cells were incubated with 100 µL of DCFH-DA solution for 1 hour and then incubated for 1 hour with 200 µM H_2O_2. The cells were lysed with cell lysis buffer, centrifuged, and the supernatant analysed with a fluorometric plate reader at 480 nm Excitation/530 nm Emission wavelengths. The principle of the DCFH-DA assay was to measure intracellular ROS activity. DCFH-DA is deacetylated via the action of cellular esterases after diffusing into cells into a non-fluorescent DCFH, which in turn is oxidised to DCF which is highly fluorescent. This in turn gives a proportional fluorescent intensity based on the intracellular ROS levels (cellbiolabs, oxiselect intracellular ROS assay kit).

3.2.4 – HPLC analysis

HPLC-PDA (photo-diode array) detection was used to analyse the intracellular extracts of human volunteer buccal cells using a Phenomenex C_{18} column (250mm x 4.6mm i.d., 5µm) and was operated at 40 ^{o}C. Buccal cells were extracted thrice with 200 µL of methanol, dried under nitrogen gas, and re-suspended in 50:50 mobile phase A and B. Peak areas were measured at 270nm and UV spectra were recorded from 190nm to 370nm on the HPLC system (Shimadzu, Japan). The system consisted of a SIL-20AC auto-sampler, an LC-

20AD liquid chromatogram, SBM-20A communication bus module, DGU-20AD degasser, CTO-20AC column oven, and an SPD-M20A photo-diode array detector. The equipment was operated using the Shimadzu LC solution software. The mobile phases used were (A) 0.1% phosphoric acid in water (w/v) and (B) acetonitrile. The sample injection volume was 20µL. The mobile phase gradient profile was as follows: 8% B for 2.5 minutes, increasing to 18% at 14.25 minutes, 24% at 19.5 minutes, 26% at 20 minutes, 28% at 23 minutes, and back 8% at 27 minutes. The flow rate was set at 1mL/min. The catechins in the buccal cells were identified by comparing their retention times and UV spectra with authentic standards. Concentrations were obtained using standard curves of the individual catechins.

3.2.5 – Cell culture

H400 cells (human oral squamous cell carcinoma, from the alveolar process) were routinely cultured in Dulbecco's modified Eagle's medium DMEM:HAMS F12 (1:1), supplemented with 10% Foetal bovine serum (FBS), 2 mM Glutamine, and 0.5 µg/mL sodium hydrocortisone succinate. All cells were cultured and maintained in 75 cm^2 flasks, with the environment controlled at 37 ^oC and 5 % CO_2. The cell medium was changed every 2 days (3 times per week), and cells were sub-cultured at 80 % confluence. For absorption and adsorption of catechins, 6-well plates were used and catechins were added at 100 µM with 10 µM ascorbic acid to keep the catechins stable. Catechins were stable in the presence of cells and ascorbic acid for 90 minutes. For the measurement of mitochondrial DNA (mtDNA) damage; 12-well plates were used and were incubated with catechins at 10 µM & 2 µM. Ascorbic acid was co-incubated at 5µM. Experiments were carried out 4 days post-seeding when cells were 100 % confluent. H400 cells at passage 27-39 were used for all experiments.

To analyse the cytoplasmic and membrane fractions: the cells were first trypsinised and washed with cold PBS; centrifuged at 500 x g for 5 minutes at 4 ^oC, and the pellet resuspended in 500 µL of cytoplasmic

isolation buffer (CIB). The pellet was vortexed, centrifuged at 500 x g for 5 minutes, and supernatant removed (cytoplasmic fraction). The remaining pellet was re-suspended in membrane isolation buffer (MIB), vortexed and centrifuged at 8000 x g for 5 minutes. The supernatant was removed for analysis (membrane fraction).

3.2.6 – Acquity ultra performance liquid chromatography (ULC)-MS analysis of H400 cell extracts

Analysis of catechins in H400 cell cytoplasmic and membrane fractions were carried out using an XBridge C_{18} column (5mm x 4.6mm i.d., 3.5µm) on an Acquity ULC system (Waters, UK). A Synapt-G2 HDMS (Waters) provided the mass spectrometric detection in negative ionisation electrospray mode. The sampling cone voltage was set at 35 V; desolvation temperature at 350^{o}C; and source temperature at 100^{o}C. Leucine Enkephalin (2 ng/mL) was used as a reference compound in negative ion mode (m/z 554.2648). A flow rate 0.3 mL/min was used and column temperature was maintained at 35^{o}C. The mobile phases were (A) 0.1 % Formic acid in water (w/v) and (B) 0.1 % Formic acid in acetonitrile. The sample injection volume was 3 µL. Initial conditions were 3 % B for 1 minute, increasing to 18 % B at 6 minutes, staying constant at 18 % B until 6.5 minutes, back to 3 % B at 6.6 minutes, and then stayed at 3 % B until 8 minutes. Accurate masses were obtained using Masslynx 4.1 software, with scanning across the spectrum of 50-1000 *m/z*, and concentrations were obtained using standard curves of the individual catechins.

3.2.7 – Analysis of mtDNA damage

H400 cells were cultured in 12-well plates to 100 % confluence. They were then washed twice with warm PBS, and incubated with 10 µM and 2 µM catechins for 1 hour and 3 hours in culture medium. Cell culture medium was removed, cells washed twice with PBS, and mitochondrial DNA (mtDNA) damage was induced by exposing the cells to 1 mM H_2O_2 for 24 hours. The H_2O_2 was removed and genomic DNA was extracted using a cultured mammalian cells Genomic DNA purification

kit (ThermoScientific). The total assay volumes for PCRs was 25µL, which included 40ng of extracted DNA, forward and reverse primers, and a SYBR green PCR master mix. The 7500 RT-PCR sequence detection software (version 1.3.1, Applied Biosystems) and Applied Biosystems 7500 RT-PCR machine was used for the assays. Mitochondrial DNA damage was expressed as a C_t value (cycles to threshold) fold difference of a long (972bp) mtDNA fragment in relation to a short (83bp) fragment mtDNA. The short fragment was undamaged at 1mM H_2O_2 and acted as an internal standard. The Ct value is inversely proportional to the quantity of DNA and is measured logarithmically as the amount of DNA is theoretically doubled in every PCR cycle.

The primers used were as follows:
Long fragment (972bp) – AL4_F (forward): 5'-CTGTTCTTTCATGGGGAAGC-3' AS1_R (reverse): 5'-AAAGTGCATACCGCCAAAAG-3'
Short fragment (83bp) – IS1 (forward): 5'-GATTTGGGTACCACCCAAGTATTG-3'
IS2 (reverse): 5'-AATATTCATGGTGGCTGGCAGTA-3'. The PCR program for the long fragment amplification was as follows: 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 55 seconds. For the short fragment amplification the PCR program was: 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 15 seconds.

3.2.8 – Statistical analysis

All data were analysed using GraphPad Prism 5.03 (GraphPad software Inc. La Jolla, California). Values were expressed mean ± SD and represented as a difference in C_t values in comparison to untreated controls. A one-way ANOVA with Newman-Keuls multiple comparison

tests were carried out to measure statistical significance. Differences were considered significant if *p* values were <0.05.
For the antioxidant capacity in human volunteer oral cells, students t test was used to measure statistical significance (n=9).

3.3 – Results

3.3.1 – Antioxidant capacity and absorption of catechins in human volunteer oral cells

The antioxidant DCFH-DA assay showed that ROS levels were significantly reduced in the green tea treated compared to baseline control cells. A significant decrease in ROS levels was observed after green tea was left in the mouth or consumed over 2.5 minutes (p=<0.05). There were also significant reductions in ROS levels at 5 minutes (p=<0.01), 10 minutes (p=<0.01), and 15 minutes (p=<0.01) (Figure 3.1). The largest reduction in ROS levels was observed between 2.5 and 5 minutes. From 5 to 10 minutes of incubation there was further slight decrease in ROS levels, but there was no further significant reduction after 10 minutes of incubation.

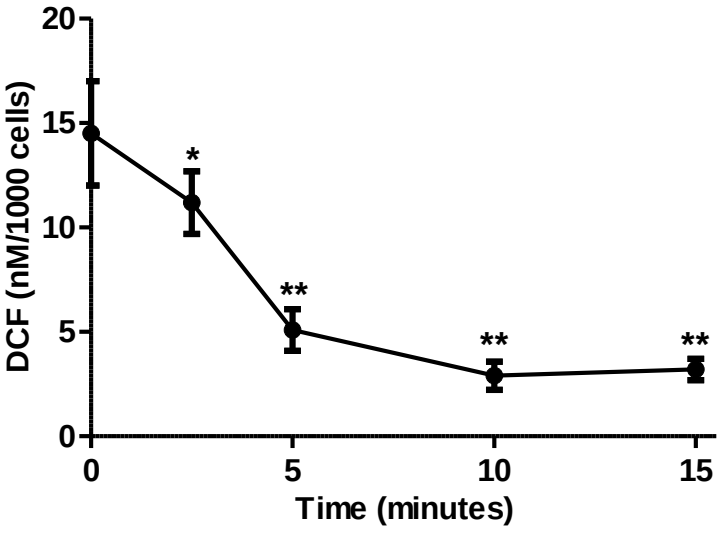

Figure 3.1 –Reduction in ROS levels in oral buccal cells after green tea incubation in the oral cavity. The results are compared to the baseline cells at 0 minutes with no exposure to green tea. Results are expressed as mean ± SEM, n=9.

Catechin concentrations were obtained through HPLC analysis of buccal cell extracts in combination with HPLC standard curves. After 2.5 minutes of green tea incubation only ECG was detected among all samples. EGCG was only detected in 1 sample after 5 minutes of exposure. However, after incubating green tea for 10 minutes there was a large increase in uptake of both ECG and EGCG (Figure 3.2), however this may have also meant that the 2 compounds became detectable under these conditions at this time point. At 15 minutes, ECG showed no further increase in absorption, whereas EGCG was increasingly absorbed in a time-dependant manner. In all samples and all time-points, EGC and EC were not detected.

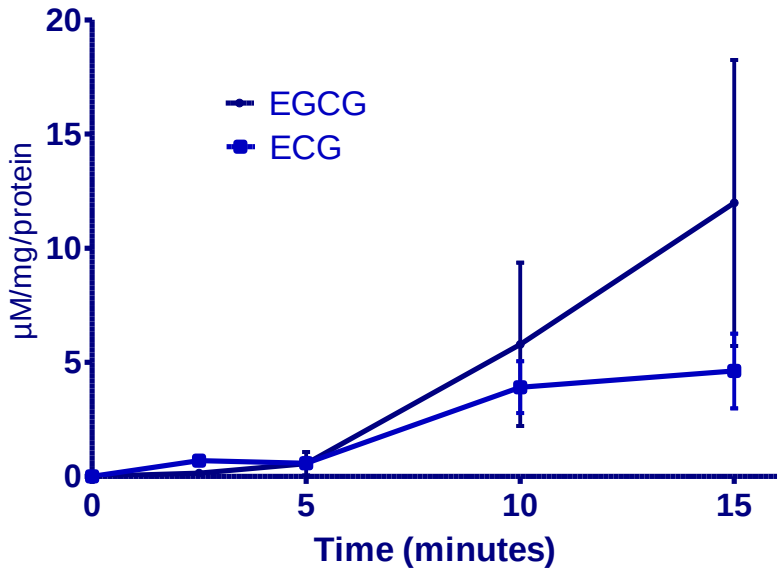

Figure 3.2 – Absorption of catechins in human volunteer buccal cells after incubation of green tea in the oral cavity for 2.5-15 minutes. Values are standardised with mean ± SD, n = 9.

3.3.2 – LC/MS analysis of H400 cells

Catechins were analysed via HPLC-MS after incubation with H400 cells for 10-60 minutes. In agreement with the results from the human volunteer samples, there was significant uptake of the gallated catechins EGCG and ECG. Both these catechins were rapidly absorbed

until the 20 minutes time-point, and then plateaued off from 30 until 60 minutes (figure 3.2). ECG was absorbed into the buccal cells at peak levels of 11.22 µM at 60 minutes and EGCG also reached a peak concentration at 60 minutes (9.08 µM). The concentration absorbed into oral cells in cell culture was very similar for the first ten minutes, after which EGCG absorption slowed down and plateaued seemingly earlier than ECG (Figure 3.3).

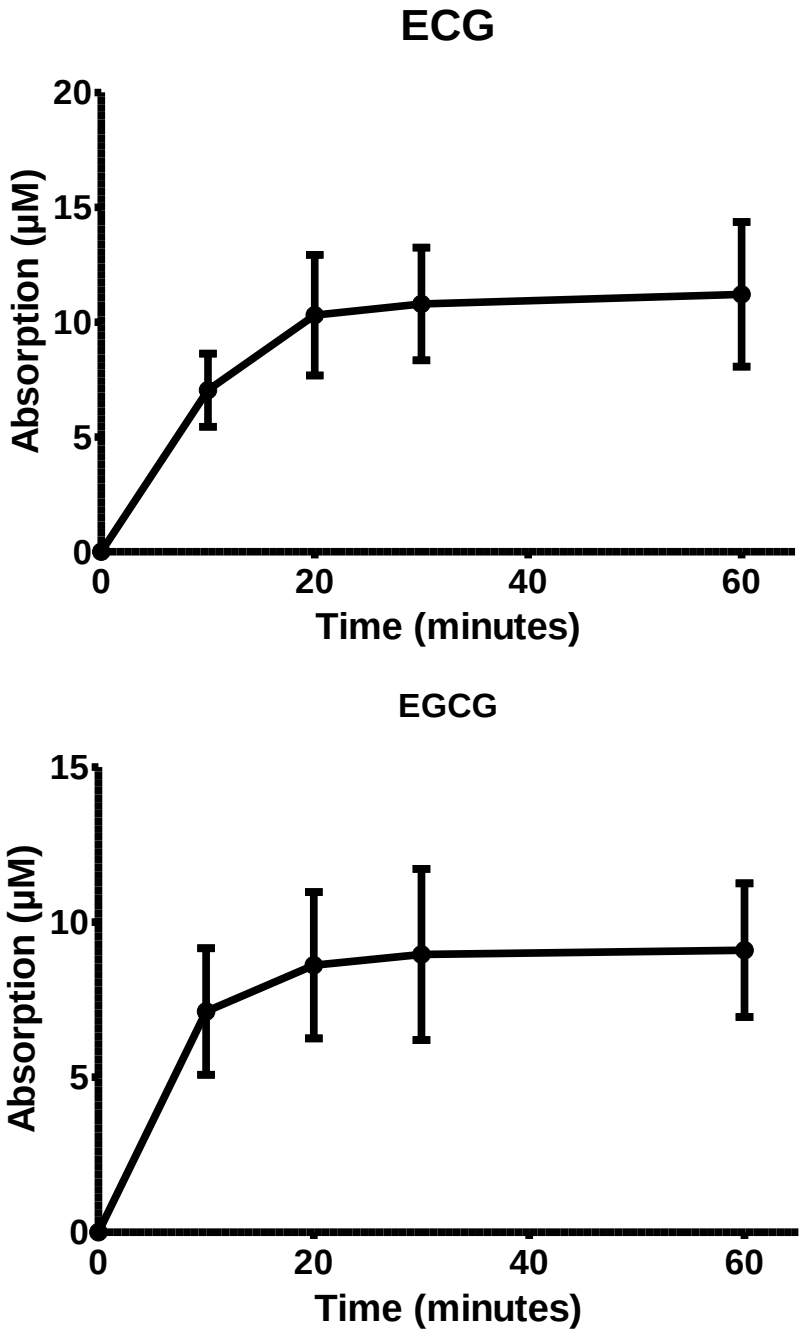

Figure 3.3 – Absorption of EGCG and ECG in oral H400 cells. Concentrations are expressed as µM/well in 6-well plates with the cell layers at 100% confluency. Values are means ± SD, n=6 (over 2 experiments in triplicate).

The results also showed the comparatively lower uptake of the non-gallated catechins in the oral cells as compared to EGCG and ECG. However, due to the increased incubation times when using the H400 cells and increase in detection limits of the UPLC-MS compared to diode array HPLC, EGC and EC were also detected, albeit in smaller but still significant quantities (figure 3.5). Cell membranes were also analysed via UPLC/MS to locate the proportion of catechins that were located on the cell surface and membrane compared to the cell plasma and cell organelle. No catechins were detected in the membrane fraction using UPLC/MS. Furthermore, no metabolites of catechins were found in the oral cells (both volunteer buccal cells and H400 cells) via analysis of a full m/z scan or 50-1000 m/z (figure 3.4).

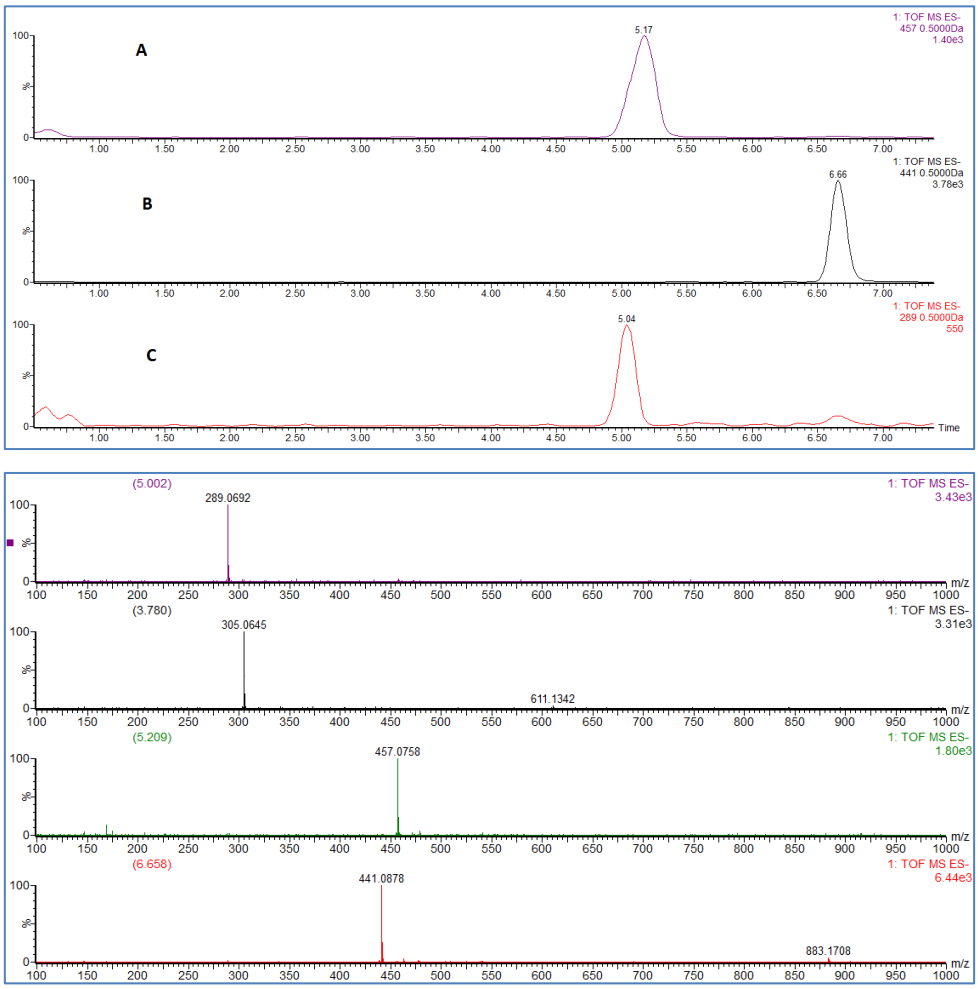

Figure 3.4 – LC chromatograms of EGCG (A), ECG (B), and epicatechin (C). The m/z peaks on the bottom half show parent catechins, with 2 peaks of epigallocatechin and epicatechin-gallate dimers (m/z 611.1342 and 883.1708 respectively). The m/z scan from 100 – 1000 m/z shows no metabolites for any of the parent catechins in any of the oral cell extracts, confirming the lack of phase 2 metabolism of catechins in the oral cavity.

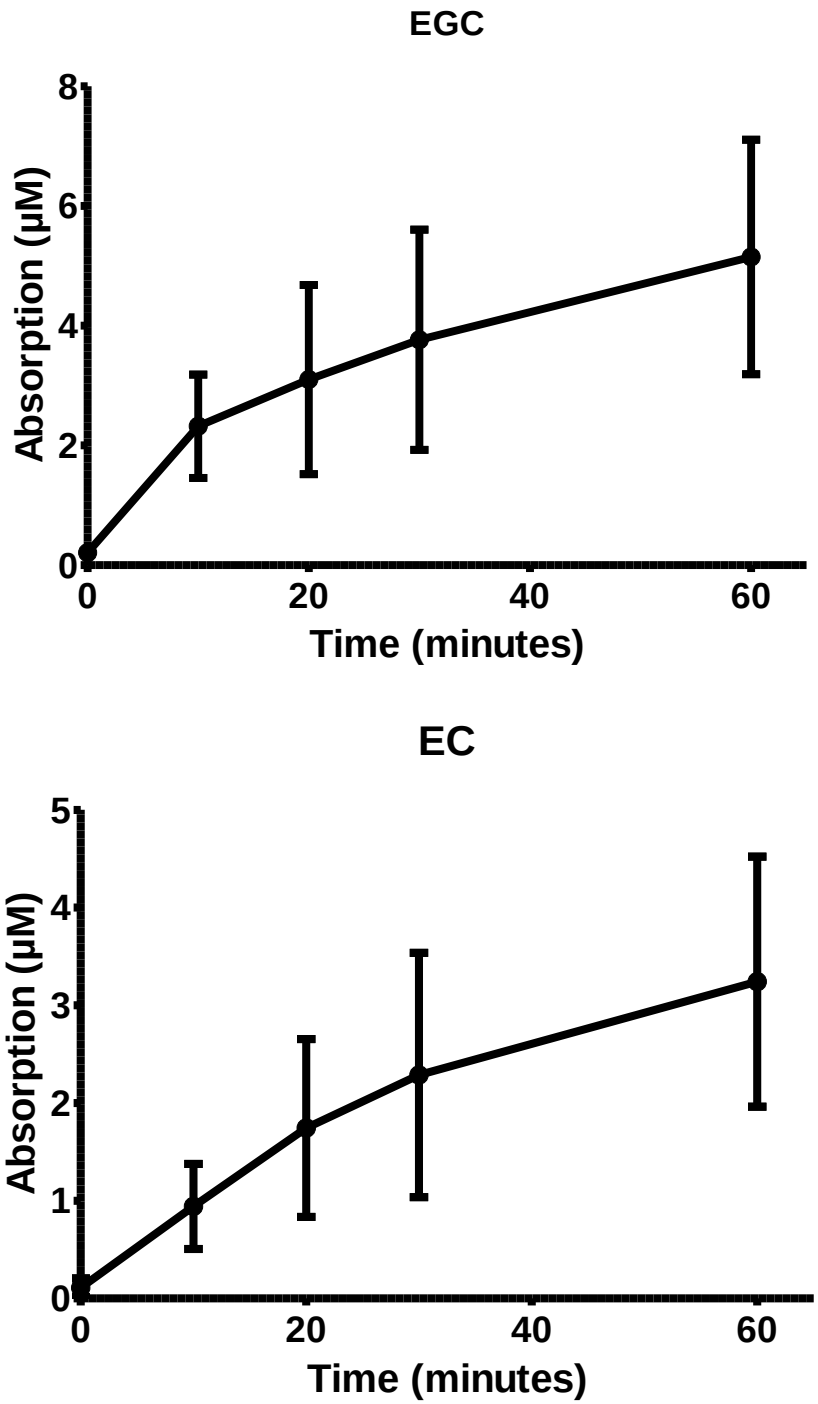

Figure 3.5 – Absorption of EGC and EC in oral H400 cells. Values are means ± SD, n=3. Concentrations are expressed as µM/well in 6-well plates with the cell layers at 100% confluency. Values are means ± SD, n=6 (over 2 experiments in triplicate).

3.3.3 – Protective effects of catechins against mitochondrial DNA damage in oral H400 cells

An optimal concentration of 1 mM H_2O_2 for 24 hours was chosen for these experiments after extensive dose response experiments were

carried out to find the optimum sub-lethal dose of H_2O_2. After incubation of catechins with H400 cells for 3 hours, there was a significant reduction in mtDNA damage with all 4 catechins at 10 µM (figure 3.6 and table 3.1). At 2 µM catechin concentrations, all but EGC showed significant protection against mtDNA damage, although statistically this was also significant at 2 µM. With the protective effects seen at 3 hours of catechins incubation, the time of catechin exposure was reduced to 1 hour (figure 3.7). At this time-point the gallated catechins EGCG and ECG showed the highest protection against mtDNA damage, even at 2 µM. 10 µM EGC and EC still displayed significant protection after 1 hour (table 3.2); however this was reduced markedly when the concentration was reduced to 2 µM.

Treatment comparison (3 hours)	*% protection*	*P*
Tiron vs H_2O_2	95.8	0.001
10µM EGCG vs H_2O_2	93.9	0.001
2µM EGCG vs H_2O_2	88.5	0.001
10µM EGC vs H_2O_2	72.3	0.001
2µM EGC vs H_2O_2	35.4	0.05
2µM EC vs H_2O_2	80.4	0.001
10µM EC vs H_2O_2	83.5	0.001
10µM ECG vs H_2O_2	79.6	0.001
2µM ECG vs H_2O_2	69.6	0.001

TABLE 3.1 - H400 cultured cells pre-treated for 3 hours with catechins and Tiron (2mM). *P < 0.05, ***P < 0.001 vs. H_2O_2 treatment; 1-way ANOVA with Newman-Keuls multiple comparison test. (n=6 over 2 experiments in triplicate)

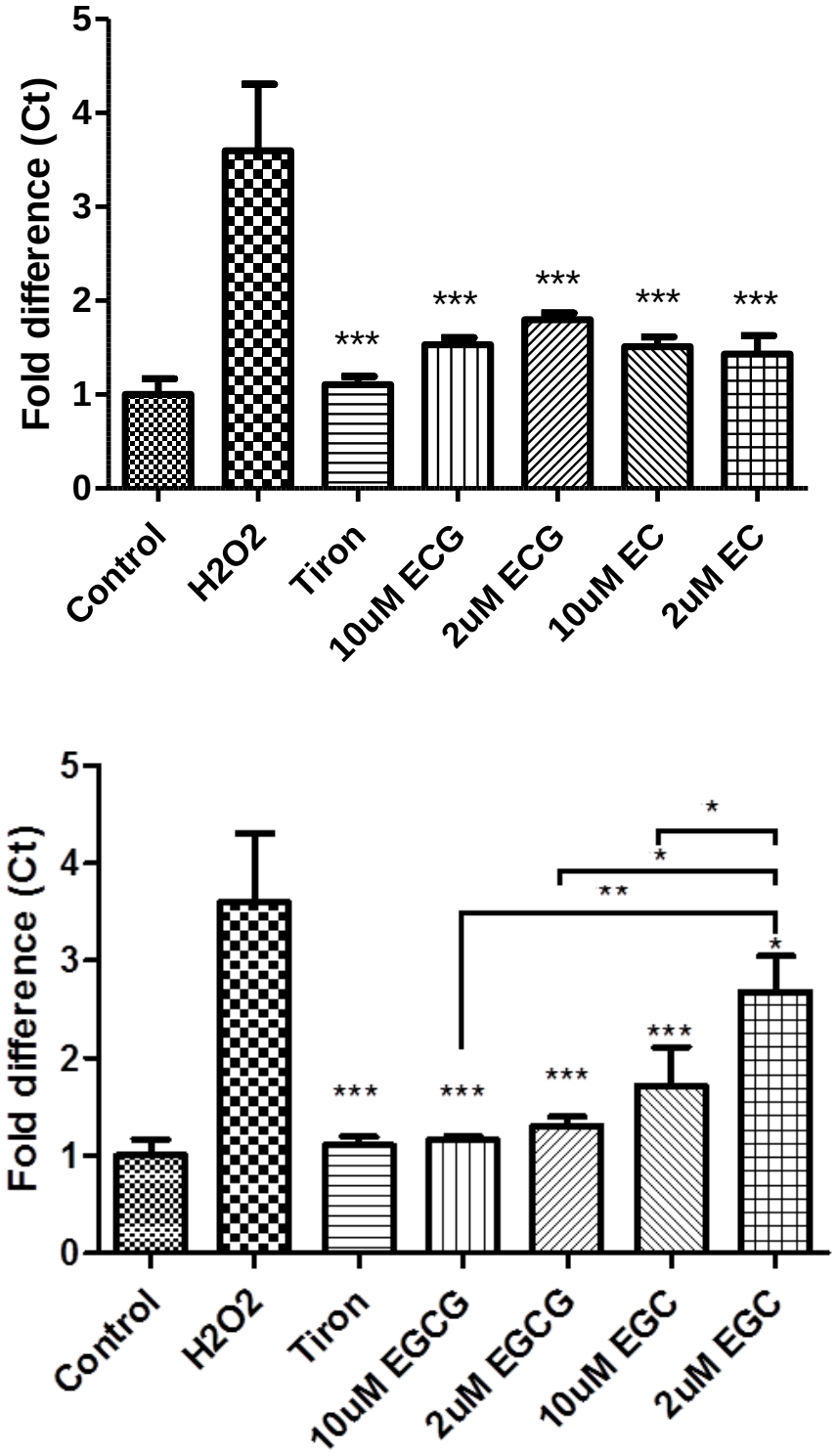

Figure 3.6 – Protective effects of 3 hours EGCG, EGC, ECG, and EC incubation against mtDNA damage in H400 cells. The damage is induced by subsequent exposure to 1mM H_2O_2 for 24 hours. The control is free from any antioxidant or oxidative damage inducer. A one-way ANOVA with Newman-Keuls multiple comparison tests were carried out to measure statistical significance of tiron and catechin treated cells in comparison to other catechins (shown on graph) and H_2O_2 treated cells. The data was normalized and values expressed as mean ± SD. *P<0.05, *** P<0.001. (n=6 over 2 experiments in triplicate)

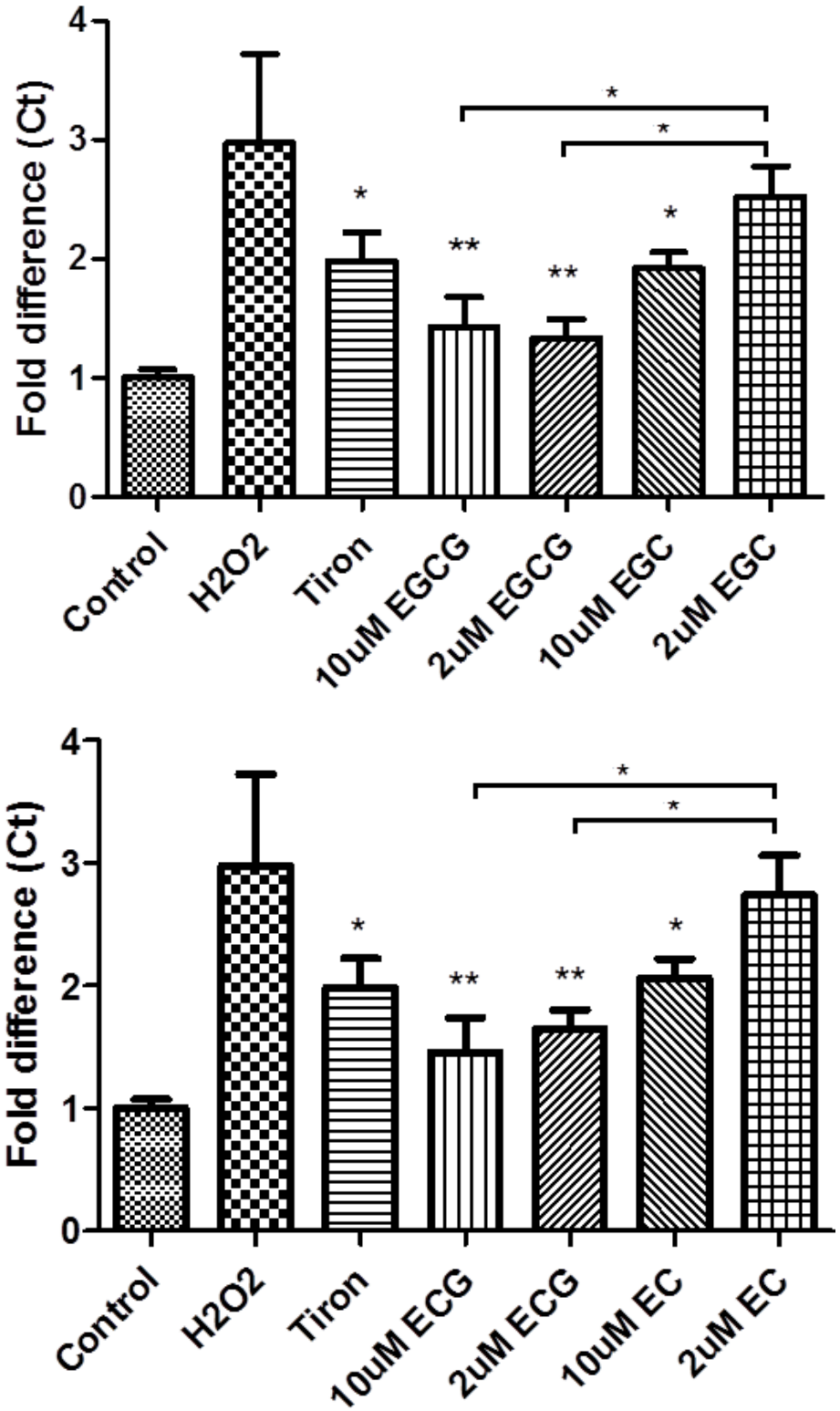

Figure 3.7 – Protective effects of 1 hour EGCG, EGC, ECG, and EC incubation against mtDNA damage in H400 cells. The damage is induced by subsequent exposure to 1mM H_2O_2 for 24 hours. The control is free from any antioxidant or oxidative damage inducer. A one-way ANOVA with Newman-Keuls multiple comparison tests were carried out to measure statistical significance of tiron and catechin treated cells in comparison to other catechins and H_2O_2 treated cells. The data was normalized and values are expressed as mean ± SD. * $P<0.05$, *** $P<0.001$. (n=6 over 2 experiments in triplicate)

Treatment comparison (1 hour)	*% Protection*	*P*
2µM EGCG vs H_2O_2	83.2	0.01
10µM EGCG vs H_2O_2	78.7	0.01
10µM EGC vs H_2O_2	53.8	0.05
Tiron vs H_2O_2	50.3	0.05
10µM ECG vs H_2O_2	76.8	0.01
2µM ECG vs H_2O_2	67.1	0.01
10µM EC vs H_2O_2	46.2	0.05

TABLE 3.2 - H400 cultured cells pre-treated for 1 hour with catechins and Tiron (2mM). *P < 0.05, **P < 0.01, vs. H_2O_2 treatment; 1-way ANOVA with Newman-Keuls multiple comparison test.

The results also confirmed that Tiron offered 100 % protection against H_2O_2-induced mtDNA damage at more than 2 hours of incubation which was shown in a previous study (Oyewole et al., 2014), and was therefore used as a positive control in all experiments. From the levels of catechins absorbed in H400 cells, there was a correlation between the levels of absorption and the degree of protection afforded against mtDNA damage. At 1 hour of incubation, EGCG and ECG were the highest absorbed catechins and also conferred the highest amount of protection against mtDNA damage. At 3 hours of incubation with catechins, EGCG showed the highest protection followed by EC and ECG. EGC was the least effective at protecting mtDNA from damage when compared with the other three catechins, but still had significant protection (*p<0.05).

4 – Discussion

The main objectives of this study were to determine the intracellular levels of catechins in oral cells and whether or not they could protect against mtDNA damage in these cells. The studies showed significant absorption of catechins into human oral H400 cells of all four catechins, and also significant protection of mtDNA in oral cells.

There are various studies which support the theory that green tea plays a significant role in maintaining good oral health. For example it was shown that dentine was protected from erosion and abrasion by rinsing the mouth with a 0.61% green tea extract (Magalhaes et al., 2009). Green tea in mouthwash was also able reduce the salivary levels of Lactobacillus and streptococcus mutans which cause caries (Ferrazzano et al., 2011). Mucins, histatins, oral peroxidases, lactoferrin, lysozyme, and secretory immunoglobulins are protective salivary components which are released indirectly from the salivary glands via green tea, which could lead to its indirect effects as an antimicrobial in the oral cavity and therefore prevent the formation of dental caries (Zhang and Kashket, 1998). Green tea was also shown to be effective against bacterial halitosis by the inhibition of formation of volatile sulphur compounds, and followed by inserting green tea catechins into treatments like mouth spray and chewing gum for bad breath (Lodhia et al., 2008, Zeng et al., 2010). It was found that salivary levels of catechins were quite high (2-260 µM) compared with plasma concentrations when a tea solution was held in the mouth or via chewing tea leaves (Yang et al., 1999b, Lee et al., 2004). Of course it was recognised that to elicit any health effects this would not be achieved via a single dose to the oral cavity; instead chewing green tea leaves to release catechins to the oral cells would be far more effective. Yang *et al* and Lee *et al* demonstrated that pharmacological concentrations could be achieved in the mouth by chewing/holding green tea leaves or tea in the mouth, effectively providing safe, cheap, and widely accepted health benefits. For aesthetic purposes lozenges or chewing gum with sustained release properties could be developed

to deliver catechins to the oral cavity and even to the stomach or intestine for example.

This is the first study to determine intracellular levels of catechins within the oral mucosa and to measure the protective effects of those catechins against mitochondrial DNA damage in the oral cavity. When samples were taken from human volunteers, there were only small amounts of samples so the amount of catechins adsorbed onto the cell membranes could not be measured; in those samples only EGCG and ECG could be detected in the buccal mucosa. The aim was to measure catechins stickiness on oral cell membranes using oral H400 cells, as polyphenols have been shown previously to be able to bind to the surface of red blood cells and microbes (Ginsburg et al., 2011, Koren et al., 2010, Koren et al., 2009). The oral cavity includes the resident microbiota, teeth, gingiva, oral mucosa, and tongue which can be possible sites for polyphenols to stick to (Schroeder and Listgarten, 1997, Dale, 2002). Aside from the possibility of adhering to the external cell membranes, a recent study demonstrated that an anthocyanin glucuronide and its microbial metabolite were detected in the oral cavity, which shows uptake and phase II metabolism as well as microbial degradation in the oral cavity (Mallery et al., 2011). There have been no previous reports of microbial or phase I/II metabolites of catechins found in the oral cavity, however Yang *et al* did find activity of a catechin esterase which cleaved EGCG into EGC in the saliva (Yang et al., 1999b). In our study there were no catechins found stuck to the H400 cell membranes which agreed with the theory that polyphenols only become 'sticky' in the presence of a synergistic effect of microbes, salivary low molecular weight antioxidants, blood cells, salivary proteins such as albumin, and other polyphenols, i.e. *in vivo* (Koren et al., 2010, Ginsburg et al., 2012, Ginsburg et al., 2013). However, significant quantities of catechins were detected in the intracellular fractions of H400 cells, suggesting that saliva and microorganisms play a minimal role in absorption of green tea catechins in the oral cavity. Due to the lack of salivary catechin esterase in cell culture, a lack of EGC in the EGCG incubation was observed. UGT1A10 is an extrahepatic phase II

enzyme found in the oral cavity but is not one of the UGTs involved in metabolising green tea catechins (Strassburg et al., 1999, Tukey and Strassburg, 2000, Tukey and Strassburg, 2001, Feng, 2006). Based on previous studies using salivary catechins and from the levels of catechins that were measured in H400 cells, it is clear that the oral cavity is exposed to unchanged parent catechins which act as very powerful antioxidants (Lambert and Elias, 2010, Henning et al., 2004, Khan et al., 2008). The results from our antioxidant capacity study demonstrate that having tea in the mouth for even 2.5 minutes reduces the ROS levels in the oral buccal mucosa significantly. These catechins can, if individuals drink green tea regularly (3-4 cups daily, or chew tea leaves) act as first line of defence against ingested pathogens, which not only has beneficial implications for good oral health but downstream health benefits for a healthy gastrointestinal tract. Mitochondrial DNA (mtDNA) is highly susceptible to oxidative damage because mitochondria are the major site of superoxide production in the cell, which is then altered into other ROS (Birch-Machin and Swalwell, 2010, Murphy, 2009). If the site of mtDNA is constantly exposed to oxidative damage constantly, this can lead to mutations in the DNA; which has been suggested as one of the principal causes of tissue ageing and carcinogenesis (Tulah and Birch-Machin, 2013). It was proposed that exposing oral cells to physiological concentrations of catechins would protect against hydrogen peroxide induced mtDNA damage, mainly because polyphenols act as powerful antioxidants which efficiently scavenge intracellular superoxide and other ROS species which can protect cells and tissues against oxidative damage (Hseu et al., 2012, Liu et al., 2011, Tobi et al., 2002, Stewart et al., 1996, Offord et al., 2002, Morley et al., 2003, Morley et al., 2005, Marrot et al., 2008, Eicker et al., 2003, Chan et al., 2003). Tiron was used as a positive control in our study as it was previously shown to be 100% effective at protecting mtDNA against oxidative damage, and that it is a mitochondria-permeable antioxidant (Oyewole et al., 2014, Supinski et al., 1999, Silveira et al., 2003, McArdle et al., 2005, Krishna et al., 1992). The oral cavity is the first point of contact for ingested materials

and therefore the first to be exposed to any beneficial or harmful compounds hence is a very important target to keep as healthy as possible. Giving green tea as a beverage or an extract can make it unclear whether the potency is because of the individual of catechins or a synergistic combination of its other constituents. In this study, H400 cells were exposed to 10 µM and 2 µM of individual catechins for 1 and 3 hours, thereafter stressed via 1 mM H_2O_2 for 24 hours to induce significant amounts of mtDNA damage. After 3 hours of incubation EGCG was found to be the most effective at both 10 and 2 µM at protecting against mtDNA damage, followed by EC, ECG, and EGC, which also had very significant amounts of protection. This correlated well with antioxidant capacity as EGCG was shown to be the most effective antioxidant of the major catechins in tea (Stewart et al., 2005). The time was then reduced further to 1 hour to make conditions as physiological as possible, but the concentrations were kept the same. Even at 1 hour of catechin exposure there was very significant protection with both 10 and 2µM of EGCG and ECG, and significant protection observed with only 10µM of EGC and EC. After 1 hour of exposure to 100µM individual catechins, intracellular levels of EGCG and ECG reached 9.1 and 11.2µM respectively. At the same time-point levels of EGC and EC were 5.1 and 3.2µM respectively, which could explain the higher levels of protection conferred by EGCG and ECG. Further studies should include measuring mtDNA damage in human buccal cells whilst drinking green tea as it is normally consumed instead of swishing it in the mouth or holding/chewing tea leaves. This would give the best indication of the exact physiological effects that the catechins have in the oral cavity with regards to absorption and protection against oxidative mtDNA damage. This has significant implications for general oral health and prevention of oral diseases, and provides a sound strategy for safe nutritional interventions for combating ROS mediated damage in the oral cavity.

5 – References

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Chapter 4 - Metabolism of green tea catechins by COMT, human liver, and intestinal phase II enzymes

Abstract

Background: Green tea catechins are extensively conjugated after ingestion via intestinal and hepatic phase II enzymes. These UGTs, SULTs, and COMT produce glucuronide, sulfate, and methylated metabolites. It is vital to understand the exact metabolites formed in different tissues so this can potentially be correlated with their production and bioactivity *in vivo*. This study aimed to determine the metabolism of green tea catechins over 3 hours after incubation with different phase II enzyme fractions with the purpose of producing sufficient amounts of metabolites to use in further experiments (mtDNA damage in chapter 5). **Methods:** catechins (200 µM) were incubated with phase II enzymes for up to 3 hours. Samples were removed at regular time-points to measure the exact amount of metabolites formed over that period. **Results:** Catechins were not extensively sulfated using s9 or liver cytosolic fractions. The major sulfates produced were 3'-O-methyl-EC-sulfates and 4'-O-methyl-EGC-sulfates. EGCG and EGC were significantly methylated via porcine COMT, and EC was metabolised to a lesser extent. Glucuronidation was the major conjugate forming pathway in our experiments. Methylation of EGCG at the 4' position (4'O-methyl-EGCG) and methylation of 4'-O-methyl-EGC at the 4' position greatly increased glucuronidation via liver microsomes. 4'-O-methyl-EGC was also sulfated much more efficiently than EGC. When EC was methylated at the 3' position, its capacity for sulfate conjugation greatly increased. **Conclusions:** The results from our metabolism experiments showed extensive glucuronidation of catechins, and also significant amounts of methylation. These can be used as preliminary studies to plan more extensive metabolism experiments in future.

4.1– Introduction

In recent years the polyphenolic catechin compounds in green tea have been widely studied for their health benefits. The observed health effects of these flavonols include the prevention of cardiovascular diseases, diabetes, neurodegenerative diseases, and cancer (Yang et al., 2006, Lambert and Elias, 2010, Higdon and Frei, 2003). The catechins account for 30-40% of the dry weight of which the main ones are; Epigallocatechin-3-gallate (EGCG), Epicatechin-3-gallate (ECG), Epigallocatechin (EGC), and Epicatechin (EC) (Balentine et al., 1997). Many studies have used intact forms of catechins *in vitro* to assess their potential effects on health; however this is not the case *in vivo* where extensive metabolism occurs via glucuronidation, sulfation, and methylation (Yang et al., 1998, Van Amelsvoort et al., 2001, Stalmach et al., 2009, Lee et al., 2002). These studies showed that catechins as well as their metabolites occurred in urine and plasma, and reached micromolar concentrations. This has been confirmed *in vitro* where the UGT-mediated glucuronidation (Vaidyanathan and Walle, 2002, Lu et al., 2003a, Crespy et al., 2004), sulfation via Sulfotransferases (Vaidyanathan and Walle, 2002, Feng, 2006), and methylation via COMT (Zhu et al., 2000, Lu et al., 2003b) of catechins were studied.
As there are so many different metabolites formed from catechins, it is of utmost importance therefore to understand the precise metabolism of these catechins to determine their bioactivities. EGC and EC, the non-gallated catechins are usually found in the plasma in their conjugated forms (69 % and 79 % respectively), with major products being 4'-O-methyl-EGC-sulfate, 4'-O-methyl-EGC-glucuronide, 4'-O-methyl-EGC, and 3' / 4'-O-methyl EC (Feng, 2006). Gallated catechins such EGCG on the other hand, are generally in the plasma in their non-conjugated forms (77 – 90 %), where catechol-O-methyl transferase mediated methylation occurs for a small amount of EGCG (Lu et al., 2003b). Due to extensive metabolism and poor absorption, only 0.1 – 1 % of ingested green tea catechins reach the systemic circulation in humans (Chow et

al., 2001, Zhang et al., 2004).
When compounds are metabolised, they are generally considered to be biologically inactive, or less bioactive than the parent compounds (Yano et al., 2007, Landis-Piwowar et al., 2008, Duenas et al., 2010), and are effectively marked for excretion via urine or bile. However, a few studies have shown that these metabolites of drugs and dietary compounds can also remain biologically active, and in some cases exert a higher bioactivity (Monks et al., 2004, Harris et al., 2004, Bai et al., 1999, Sano et al., 1999, Koga and Meydani, 2001, Chiu and Lin, 2005).
For this reason, the determination of precise metabolism of green tea catechins is of vital significance, as more comprehensive data will make it easier to correlate the different metabolites to their potential *in vivo* health effects. As the detailed ADME properties of catechins are extremely important to increase our understanding of their mechanisms of action *in vivo*, and their overall contribution to the prevention of chronic diseases.

To our knowledge, the metabolism of the four major green tea catechins over the first 180 minutes of incubation with phase II conjugating enzymes has not yet been investigated comprehensively *in vitro*, in human liver and intestine. With this gap in the data, and to produce metabolites for further experimental use, experiments were carried out to quantify the biotransformation of the major catechins using different phase II enzyme fractions from human liver and intestine, along with Catechol-O-methyl transferase (COMT) from porcine liver.

4.2 – Materials and methods

4.2.1 – Chemicals and reagents

The following were purchased from Sigma Aldrich (Gillingham, UK): Dithiothreitol (DTT); Magnesium Chloride ($MgCl_2$); S-(5′-Adenosyl)-L-methionine (SAM); Ascorbic Acid; Catechins (EGCG, EGC, ECG, & EC);

Molecular biology grade H_2O; 0.5 Molar Tris-HCl buffer; and Adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (PAPS). ULC/MS-grade Acetonitrile; ULC/MS-grade methanol; Acetic acid; and Formic acid were purchased from Biosolve BV (Valkenswaard, the Netherlands). Purified Catechol-O-Methyl Transferase (COMT) from Porcine liver was purchased from Calzyme laboratories, Inc. (San Luis Obispo, California). Human liver microsomes (HLM), human intestinal microsomes (HIM), human liver S9 fraction, human liver cytosol, UGT reaction mix A (25 mM UDPGA), and UGT reaction mix B (250 mM Tris-HCl buffer, 40 mM $MgCl_2$, 0.125 mg/mL Alamethicin) were purchased from BD biosciences (Swindon, UK).
3'-O-methyl Epicatechin (Me-EC), 4'-O-methyl Epigallocatechin (Me-EGC), and 4'-O-methyl Epigallocatechin gallate (Me-EGCG) were purchased from Mitsui Norin Co. Ltd (Shizuoka, Japan).

4.2.2 - Glucuronidation of Catechins

Glucuronide assays were carried out using the manufacturer's instructions (BD biosciences). Molecular biology grade H_2O, UGT reaction mix A, UGT reaction mix B, 200 µM catechin substrate, and 50µM ascorbic acid were combined in a total volume of 1 mL and warmed to 37 ^oC for 5 minutes. The reactions were initiated using differing amounts of protein depending on the cellular fractions used; 1 mg of liver S9 fraction, 0.2 mg of human intestinal microsomes (HIM), and 0.5 mg of human liver microsomes (HLM). Samples of 100 µL were removed at 0, 10, 20, 30, 60, 90, 120, and 180 minutes and added to 100 µL of ice-cold methanol containing 1 % ascorbic acid and acetic acid. After centrifugation at 10000 g for 10 minutes the supernatants were analysed via LC/MS.

4.2.3 - Sulfation of Catechins

Catechin sulfation was carried out using BD biosciences supplier manuals for liver S9 fraction and human liver cytosol. A 1 mL reaction mixture containing molecular biology grade H_2O, 500 mM tris-HCl buffer,

2 mM PAPS, 200 µM catechin substrate, and 50 µM ascorbic acid was warmed to 37 ^{o}C. The reaction was initiated by adding either 0.5 mg of liver S9 protein or 0.2 mg of liver cytosolic protein. The reactions were terminated by adding 100 µL to an equal volume of ice-cold methanol containing 1 % ascorbic acid and acetic acid. After thorough vortexing and centrifugation the supernatants were analysed via LC/MS.

4.2.4 – Methylation of Catechins

Catechins were methylated using a similar method to Lu *et al* (Lu et al., 2003b). An incubation mixture containing 10 mM tris-HCl, 1 mM dithiothreitol, 2 mM $MgCl_2$, 200 µM SAM, 50 µM ascorbic acid, and 200 µM catechin substrate was warmed to 37 ^{o}C for 5 minutes. Purified porcine liver COMT (500U) or human liver cytosol (0.1 mg) was added to the mixture to initiate the reaction. The total reaction mixture was carried out in a final volume of 1 mL. 100 µL aliquots were removed at 0, 10, 20, 30, 60, 90, 120, and 180 minutes; at each time-point the reaction was stopped using an equal volume of 1 % ascorbic acid and acetic acid in ice-cold methanol. The aliquots were centrifuged and the supernatants were analysed for metabolites via UPLC/MS.

4.2.5 – Data acquisition

Analysis of catechins and their metabolites was carried out using mass spectrometric detection in negative ionisation electrospray mode on an Acquity UPLC system and Synapt-G2 HDMS (Waters, UK). Separation was acquired via an Xbridge C_{18} column (50mm x 4.6mm i.d., 3.5 µm) at a flow rate of 0.3 mL/min; and column temperature was maintained at 35 ^{o}C. The sample injection volume was 3 µL. The sampling cone voltage was set at 35V; capillary voltage was set at 2.2 kV; desolvation temperature at 350 ^{o}C; lockspray capillary voltage was set at 3 kV; and source temperature at 100 ^{o}C. Leucine Enkephalin (2 ng/mL) was used as a reference compound in negative ion mode (*m/z* 554.2648). The mobile phases were (A) 0.1% Formic acid in water (w/v) and (B) 0.1% Formic acid in acetonitrile. Initial conditions were 3% B for 1 minute,

increasing to 18% B at 6 minutes, staying constant at 18% B until 6.5 minutes, back to 3% B at 6.6 minutes, and then stayed at 3% B until 8 minutes. Accurate masses were obtained using Masslynx 4.1 software, with scanning across the spectrum of 50-1000 *m/z*. For HPLC-DAD the separation was acquired via an Xbridge C_{18} column (50mm x 4.6mm i.d., 3.5 µm) at a flow rate of 0.3 mL/min; and column temperature was maintained at 35 ^{o}C. The sample injection volume was 3 µL. Wavelengths were measured in the range of 220 – 450 nm.
Due to the lack of catechin metabolite standards, quantification was done relative to the parent catechins in the assays based on both UV-DAD and MS intensity signals.

4.3– Results

To analyse the metabolism of the four major catechins found in green tea, the studied flavan-3-ols were incubated with human phase 2 enzymes (UGTs and Sulfotransferases) and porcine liver COMT for up to 180 minutes. Length of incubation was determined by the stability of the catechin in the enzyme mixture. Samples were removed at 0, 10, 20, 30, 60, 90, 120, and 180 minutes, which were extracted and analysed via diode array ULC/MS with electrospray ionisation using a MS^e acquisition method to quantify catechin metabolites and their parent compounds.

EGCG & 4'-O-Methyl-EGCG

The production of metabolites of EGCG, and its degradation by-products under the specific enzyme assays are shown in figures 4.1 – 4.5. Glucuronide, sulfate, and methylated conjugates of EGCG were observed in all enzyme fractions. Eleven metabolites were detected in total, two of which were methylated metabolites (M1 & M2), seven glucuronides (G1-7), and two sulfates (S1 & S2). Two glucuronides, one sulfate, and one methylated metabolite had a later retention time than EGCG. The remaining seven conjugates had an earlier retention time than EGCG. There were no methylated metabolites detected in the presence of liver cytosol. COMT from porcine liver produced two methylated metabolites named here as M1 and M2. These were identified by their molecular weight which had m/z values of 471.0981 and 471.0648. Here, the mass error was too high (333 ppm) for proper identification and may have been due to instrument error. M1 appeared to be the major metabolite formed based on the MS/MS and UHPLC data and reached a concentration of 23.15 ± 5.8 μM after 180 minutes, whereas M2 reached 9.27 ± 2.83 μM (Figure 4.1). Small amounts of an EGCG dimer (m/z 915. 1578) were also detected.

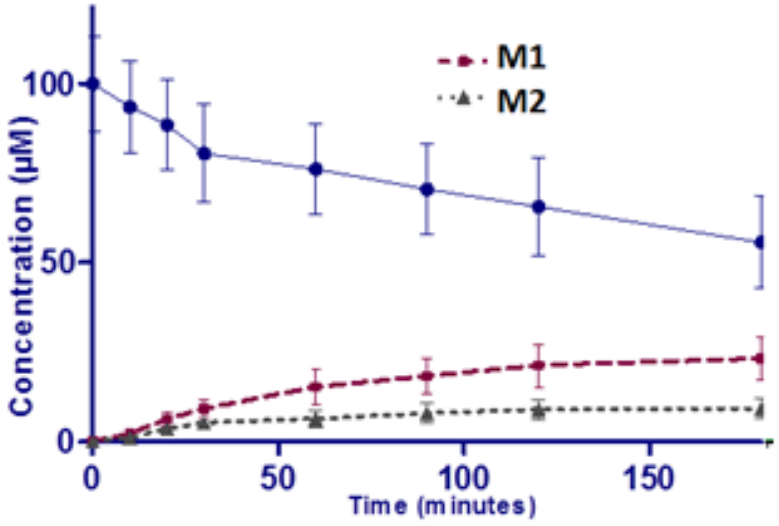

Figure 4.1 – formation of EGCG methylated metabolites after incubation with COMT from porcine liver for up to 180 minutes. Values are expressed as mean ± SD, n=6. M1 and M2 are the methylated metabolites formed.

Incubation of EGCG with human liver microsomes and S9 fraction yielded the four glucuronide metabolites G1-4 (figure 4.2). These were identified as glucuronides with a molecular weight of 633. The m/z of the glucucronide conjugates G1 – G4 was 633.1046. The retention times in both fractions were identical so were assumed to be the same glucuronides. The EGCG dimer (m/z 915. 1578) was also detected in small amounts in these conditions, along with a hydroxylated glucuronide (m/z 649.1069) conjugate which as far as is known has not been reported in previous studies (figure 4.3). Liver S9 formed 42.48 ± 18.2 µM glucuronide conjugates after 180 minutes, and with liver microsomes 24.91 ± 7.28 µM glucuronide conjugates were formed after 60 minutes. Using human intestinal microsomes, EGCG was incubated for 90 minutes and three glucuronides (G5-7) were formed (figure 4.4), along with detectable amounts of the hydroxylated glucuronide, and an EGCG dimer previously identified as theasinensin A (m/z 913.1345). G6 was the major glucuronide formed after 90 minutes, followed by G5 and G7 (figures 4.4 and 4.5). G6 was observed to have a later retention time than EGCG and G5 and G7 were eluted earlier than EGCG. The maximum concentration of 27.5 ± 9.17 µM combined metabolites was reached at 30 minutes. After

incubation of EGCG with intestinal and liver microsomes, the metabolites of EGCG reached a peak at 30 minutes, and with liver S9 the metabolites reached their maximal point at 120 minutes.

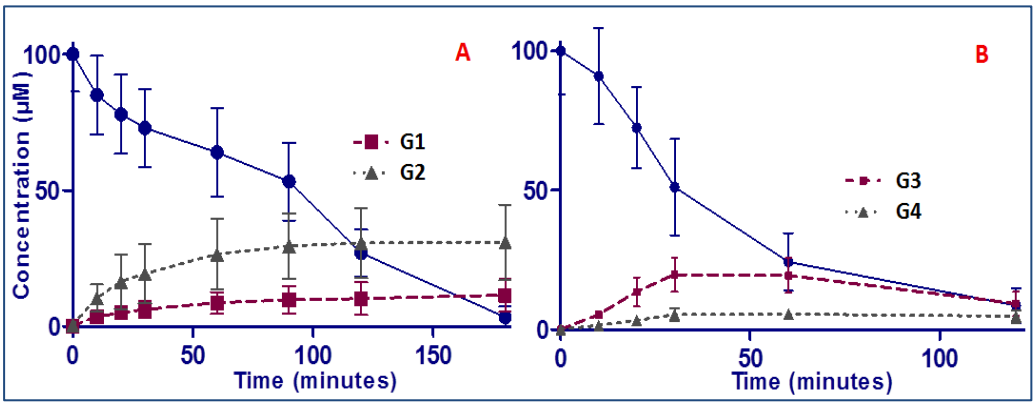

Figure 4.2 – formation of EGCG glucuronide conjugates using Human liver S9 fraction (A) and human liver microsomes (B). Values are expressed as mean ± SD, n=6. G1 – G4 are the glucuronide conjugates formed during the assay.

EGCG was sulfated by liver S9 with one product formed (m/z 537.0296), reaching a maximum of 4.1 ± 0.813 μM at 90 minutes. The parent compound was stable in the presence of the S9 fraction with >90% still present after 180 minutes. With liver cytosol, only trace amounts of one EGCG sulfate (m/z 537.0296) was observed after 120 minutes.

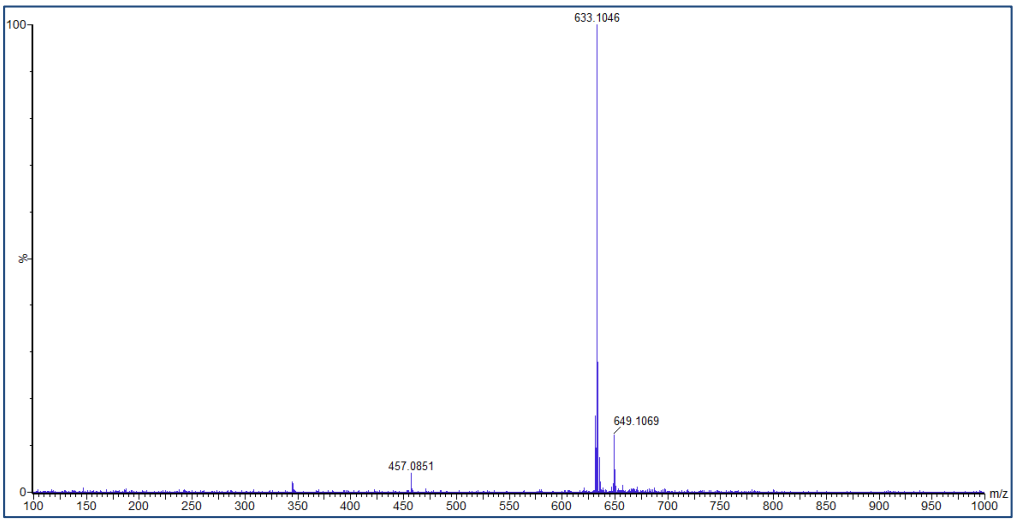

Figure 4.3 – m/z of the glucuronide conjugate G3 (m/z 633.1046), and detectable levels of the hydroxylated glucuronide (m/z 649.1069)

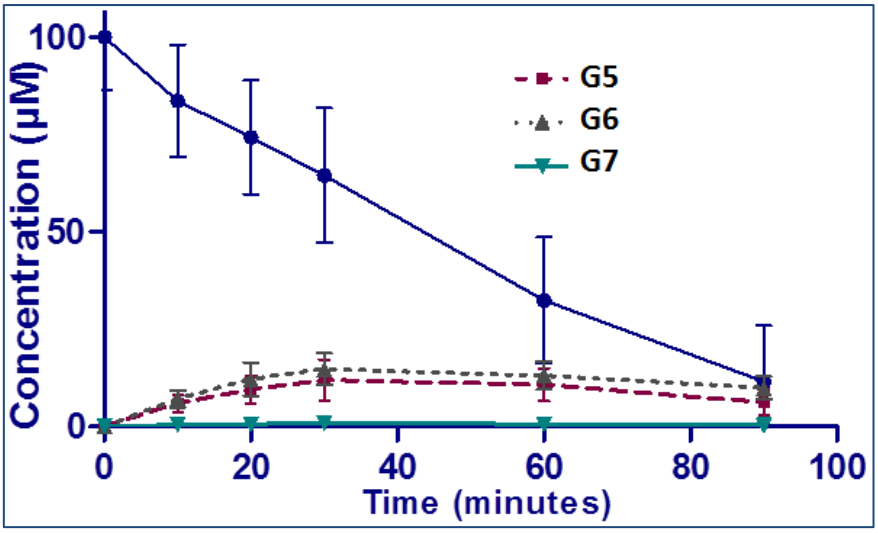

Figure 4.4 – formation of EGCG glucuronide conjugates using Human intestinal microsomes for up to 90 minutes. This time-point was lower than other metabolism experiments due to apparent instability of EGCG under these experimental conditions. Values are expressed as mean ± SD, n=6.

After observing the metabolism of EGCG, the methylated metabolite 4'O-methyl-EGCG was glucuronidated and sulfated using human liver microsomes and cytosolic fraction.

Incubation of 4'O-methyl-EGCG with liver cytosol yielded one sulfate conjugate which was detected in trace amounts after 120 minutes with an m/z 551.0464. To measure glucuronide formation the methylated EGCG was incubated with liver microsomes. After 60 minutes there were five conjugates present (figure 4.6). Three were glucuronide conjugates with molecular weights of 647.1249, 647.1267, and 647.1404. Two conjugates were found to be di-glucuronides (m/z 823.1612 and 823.1572, figure 4.7). To the best of our knowledge this is the first time di-glucuronide conjugates of methylated EGCG are reported. The major glucuronide produced was a mono-glucuronide reaching 40.36 ± 12.04 µM at 60 minutes. The di-glucuronide conjugates were the next highest metabolites formed at concentrations of 22.96 ± 4.14 µM and 11.88 ± 3.76 µM at 60 minutes. The two remaining glucuronides reached 5.8 and 4.11 µM at 60 minutes. The five glucuronides accounted for approximately 85 % of the parent compound after 60 minutes.

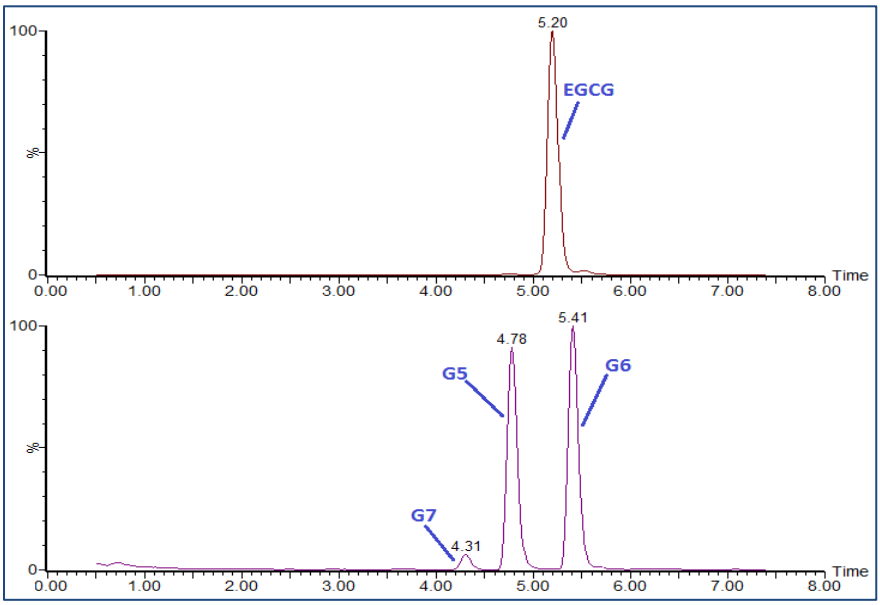

Figure 4.5 – selected total ion current (TIC) chromatogram of EGCG and its glucuronide conjugates in human intestinal microsomes

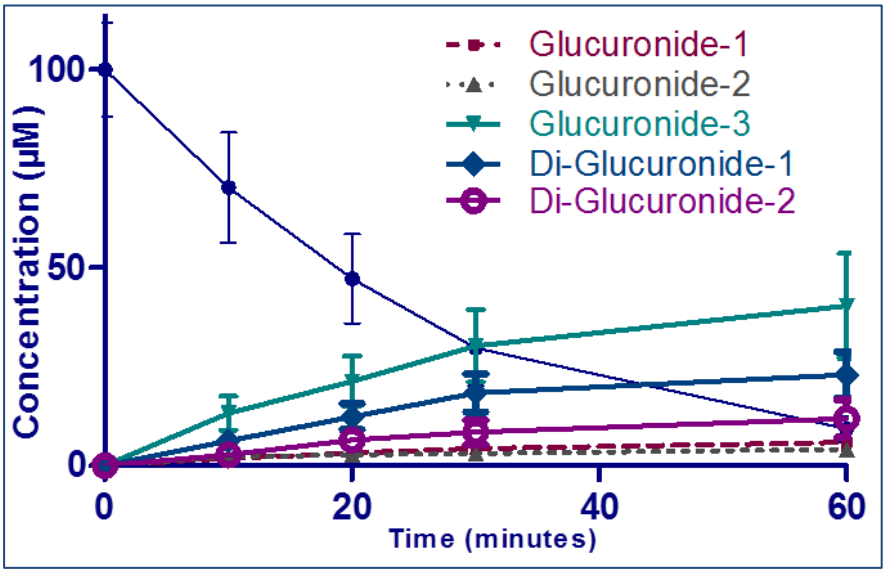

Figure 4.6 – formation of 4'-O-methyl-EGCG glucuronide conjugates using Human liver microsomes. Here, 60 minutes was enough to convert approximately 85% of the 4'-Me-EGCG into glucuronides. Values are expressed as mean ± SD, n=6.

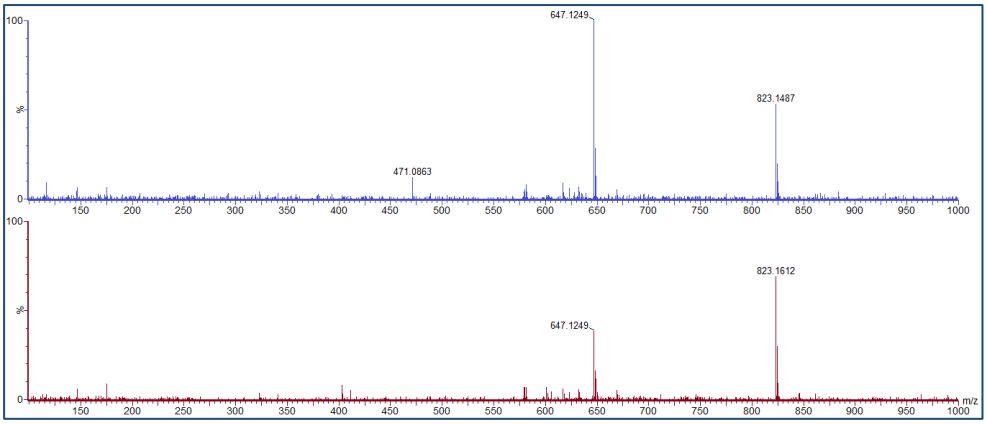

Figure 4.7 – m/z of Methyl-EGCG glucuronides (647.1249) and di-glucuronides (823.1487 and 823.1612) using Human liver microsomes.

EGC and 4'-O-methyl-EGC

Upon incubation of EGC with human liver s9 fractions and liver/intestinal microsomes, two metabolites were formed in each cellular fraction (figure 4.8). Based on retention times and *m/z* values, they were the same glucuronide conjugates so in these studies were called G8 and G9. In s9 fraction the two glucuronides reached a peak combined concentration of 7.62 ± 1.81 μM at 180 minutes, 4.93 ± 1.347 μM at 90 minutes in liver microsomes, and 6.25 ± 2.17 μM in intestinal microsomes at 90 minutes. EGC degraded at a much faster rate in the presence of intestinal microsomes and liver microsomes, but remained stable when incubated with the liver s9 fraction. Small amounts of EGC-glucuronide dimers were also present; one dimer when incubated with liver microsomes (m/z 963.2071) and one dimer when incubated with intestinal microsomes (m/z 963.1935) (figure 4.9).

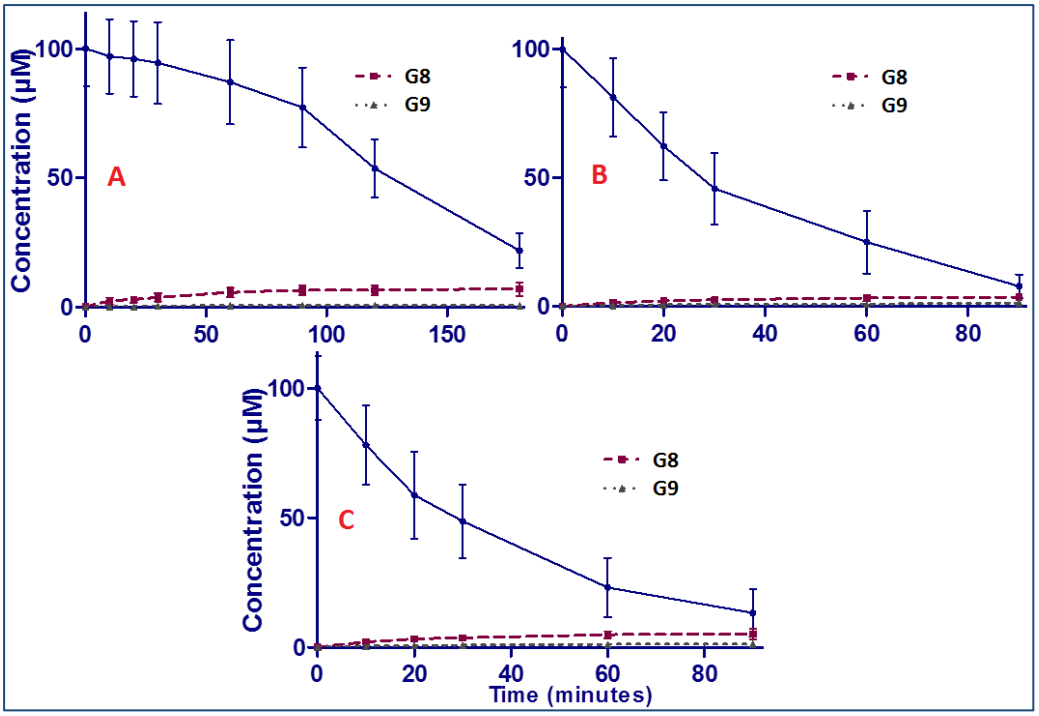

Figure 4.8 EGC glucuronides G8 and G9 formed in liver s9 fraction (A), liver microsomes (B), and intestinal microsomes (C). Values are expressed as mean ± SD, n=6.

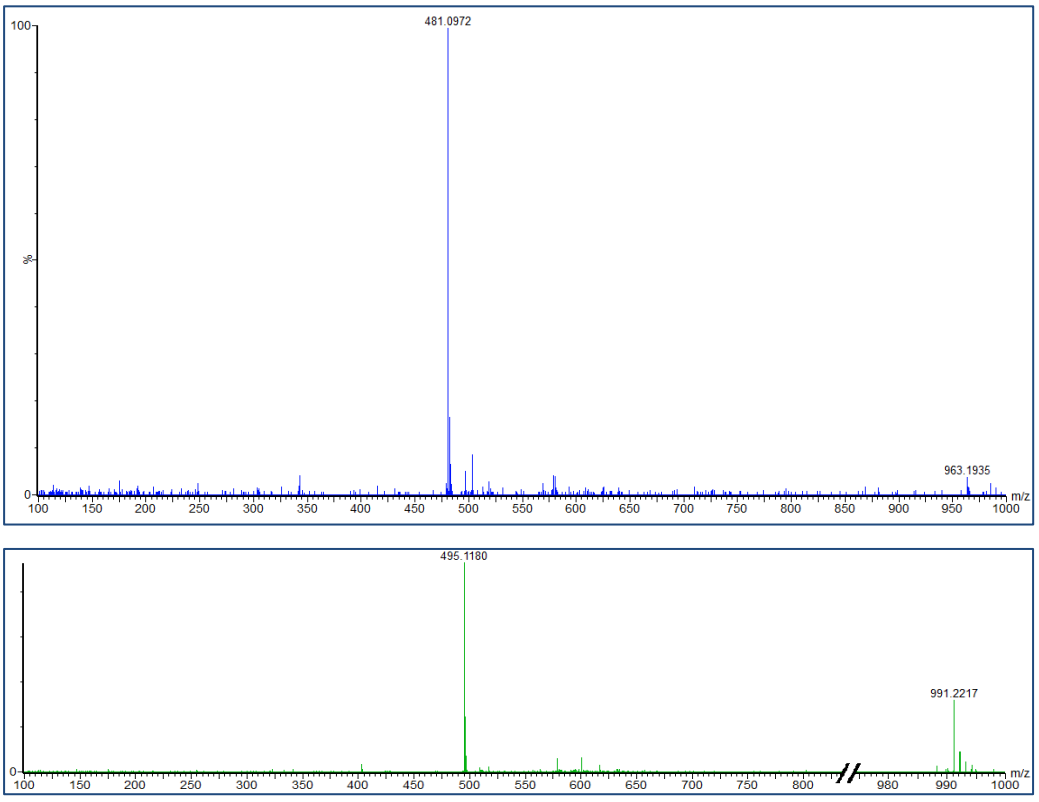

Figure 4.9 - m/z of EGC glucuronide (481.0972) and its dimer (963.1935) (blue) and figure4.10 – 4'-O-methyl-EGC-glucuronide (m/z 495.1180) and its dimer (m/z 991.2217) (green)

When EGC was incubated with COMT from porcine liver, one methylated metabolite (M3) was formed after 180 minutes with an m/z of 319.0796 (figure 4.11). The parent compound was stable under these assay conditions for up to 180 minutes and thereafter rapidly declined, and produced up to 47.3 ± 5.93 μM of the single methyl metabolite at 180 minutes. The retention time was earlier than the parent compound. As with EGCG, there was no methyl conjugates formed in the presence of liver cytosol.

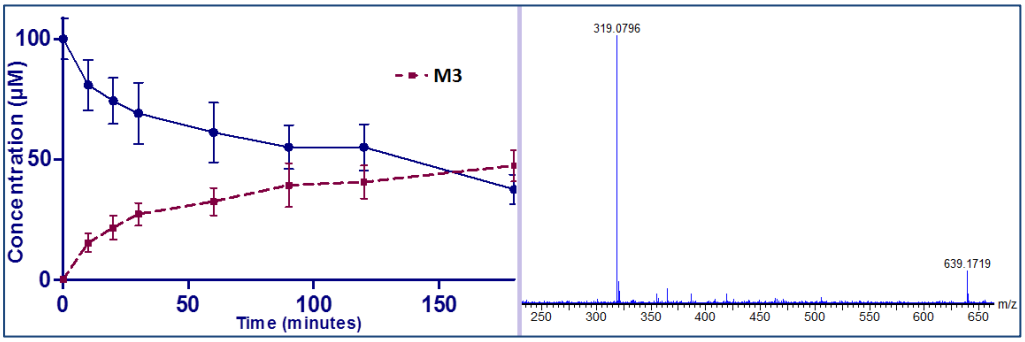

Figure 4.11 – methylation of EGC via porcine liver COMT over 180 minutes, and m/z of the methylated metabolite (319.0796) and dimer (639.1719). Values are expressed as mean ± SD, n=6.

Two sulfated conjugates (S3 and S4) of EGC were observed in liver S9 with molecular weights of 385.1874 and 385.1065. The mass error observed here made it difficult to make valid identifications of these metabolites. The parent EGC rapidly degraded after the 90 minute mark and the peak concentration of the combined sulfates reached 4.93 ± 1.098 μM. In liver cytosol the parent compound remained stable for up to 120 minutes, however only trace amounts of one EGC sulfate (S5) were present (m/z 385.0205).

4'-O-methyl-EGC was incubated with liver cytosol and liver microsomes. One glucuronide (m/z 495.1180) was formed reaching a peak concentration of 91.04 ± 10.38 μM after 60 minutes (figure 4.12). Trace amounts of 4'-O-methyl-EGC glucuronide dimer were also observed (m/z 991.2217). After 60 minutes only 7% of the parent 4'-O-methyl-EGC was remaining.

Using liver cytosol, two sulfates were observed after 180 minutes and are

shown in figure 4.13 (both with m/z 399.0405). They reached a combined peak concentration of 47.53 ± 9.60μM after 180 minutes. Both sulfates eluted earlier than 4'-O-methyl-EGC. The parent 4'-O-methyl-EGC reached undetectable levels after 210 minutes.

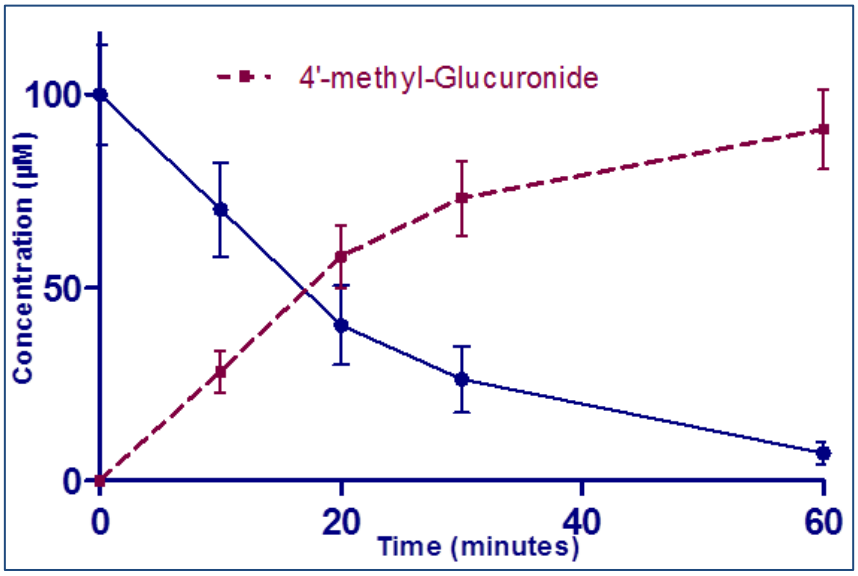

Figure 4.12 – Glucuronidation of 4'-O-methyl-EGC via human liver microsomes over 1 hour. Values are expressed as mean ± SD, n=6.

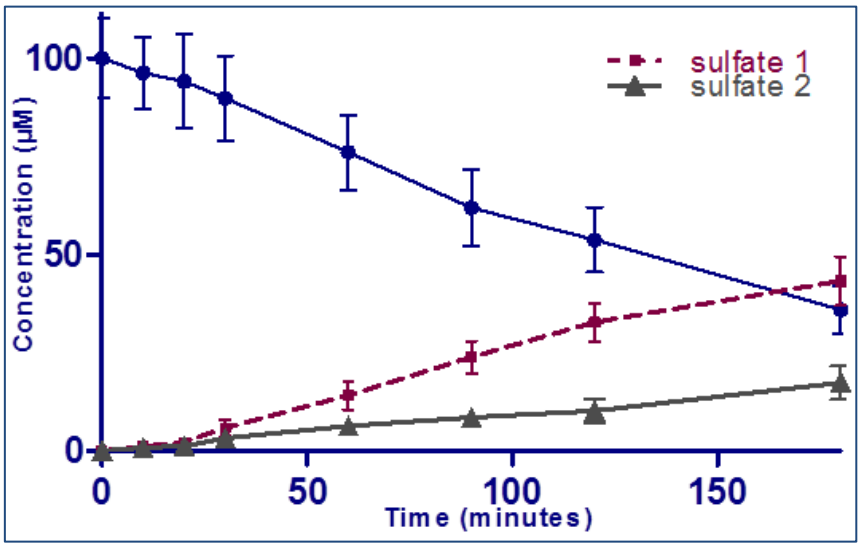

Figure 4.13 – Sulfation of 4'-O-methyl-EGC via human liver cytosolic fraction over 180 minutes. Values are expressed as mean ± SD, n=6.

ECG

There were no methylated metabolites of ECG found after incubation with porcine liver COMT and liver cytosol, and no sulfate conjugates observed in both liver S9 and cytosol incubations. No responses were observed when molecular ions for the methylated and sulfated conjugates were monitored at m/z 455 or 521Da respectively.

ECG was extensively glucuronidated in the presence of liver s9 fraction, liver microsomes, and intestinal microsomes. In both liver s9 and liver microsomes, two glucuronide conjugates were formed and are shown in figure 4.14. They are identified here only as G10 and G11. Both metabolites in both fractions had identical retention times and m/z values so were presumed to be the same glucuronide conjugates. G10 had an earlier retention time (6.09 minutes) than ECG (6.58 minutes) and G11 had a later retention time (6.90 minutes), the m/z for both metabolites was 617.1173. The peak concentration of the two combined metabolites reached approximately 93.6 µM at 90 minutes in the s9 fraction and 86.5 µM in liver microsomes after 120 minutes.

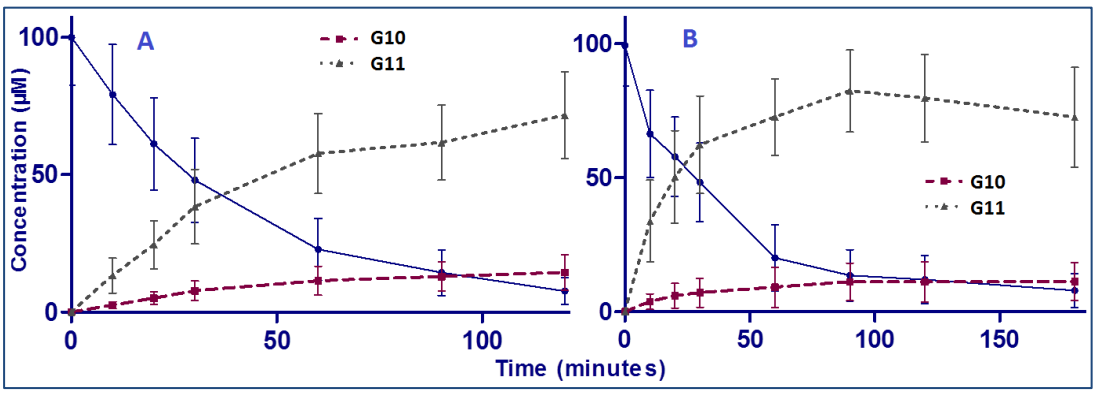

Figure 4.14 – ECG glucuronide (G10 and G11) formation from the parent ECG in liver microsomes (A), and liver s9 fraction (B). Values are expressed as mean ± SD, n=6.

Incubation of ECG with human intestinal microsomes for 180 minutes formed four glucuronide conjugates. The major metabolite was G11, identical to the one formed in liver fractions based on retention time and m/z. The three minor metabolites (G12-14) had earlier retention times than

the parent compound and are shown in figure 4.15. The glucuronide G10 was not observed to be identical to any of the three metabolites G12-14 based on retention times and m/z values. The parent ECG remained stable in all the UGT enzyme assays although small amounts of ECG dimers were observed (m/z 883.1708).

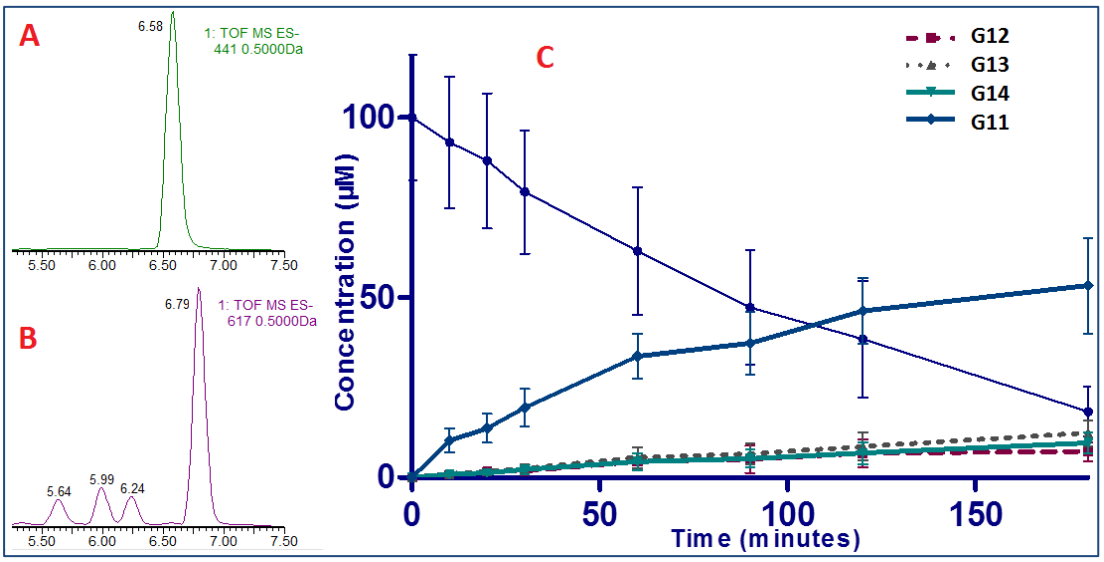

Figure 4.15 – ECG glucuronide formation in human intestinal microsomes (C), total ion chromatogram of parent ECG (A), and glucuronide conjugates (B). Values are expressed as mean ± SD, n=6.

EC

Incubation of EC with human liver microsomes and s9 fractions yielded identical metabolites in both fractions (G15 and G16) with m/z 465.1058 (figure 4.16). Both glucuronides had earlier retention times than EC. G15 was the major metabolite in both microsomal and s9 fractions. The combined glucuronides reached a peak of 19.26 ± 3.94 µM in liver microsomes and 14.02 ± 2.81 µM in the s9 fraction. In intestinal microsomes, the two same glucuronides were formed and reached a combined peak concentration of 16.07 ± 5.7 µM.

Two EC methylated metabolites (M4 and M5) were produced in the presence of COMT after 180 minutes. Both had earlier retention times than EC. The metabolites have m/z 303.0718 and 303.0711 Da and reached a combined peak concentration of 11.3 ± 2.09 µM after 180

minutes. EC remained stable in the assay up to 180 minutes. In the incubation with liver cytosol there were no methyl metabolites.

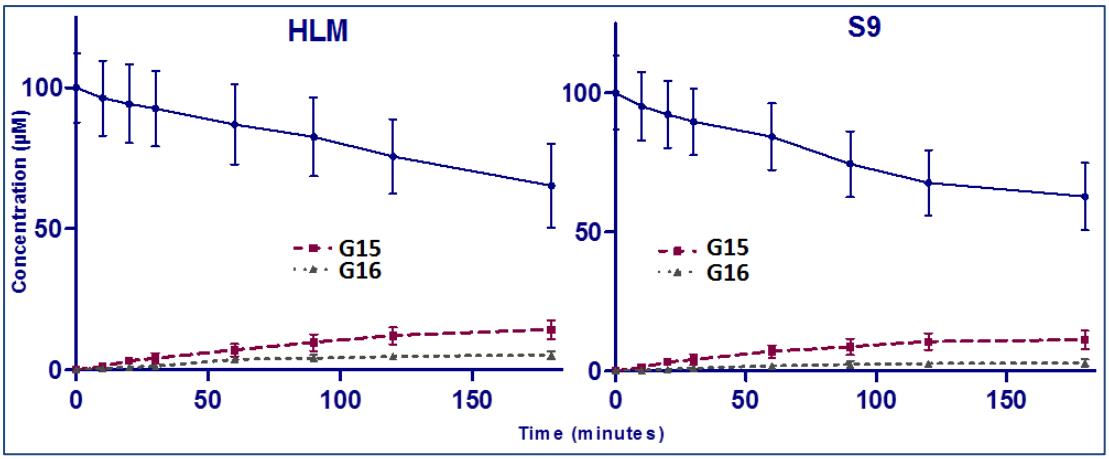

Figure 4.16 – EC glucuronide formation in human liver microsomes and s9 fraction. Values are expressed as mean ± SD, n=6.

Incubation of EC with liver cytosol yielded trace amounts of two sulfates (m/z 369.0298). Sulfation was much higher in liver s9 fraction compared to the cytosol as two sulfates reached a peak concentration of 7.18 ± 1.81 µM after 180 minutes. Both metabolites had an earlier retention time than EC. The m/z of the sulfates were 369.0318 and 369.0298, the second of which was identical to the sulfate formed via the s9 fraction. Here the error in mass was attributed to instrument error. In both incubations EC was stable for up to 180 minutes, with >70% still intact.

3'-O-Methyl-EC was incubated with HLM and formed two glucuronides in small quantities (m/z 479.1230), the combination of which reached 4.08 ± 1.14 µM after 60 minutes. One glucuronide eluted after 3'-O-Methyl-EC and one earlier.

Sulfation of 3'-O-Methyl-EC yielded two sulfate conjugates (m/z of 383.0406) with peak concentrations of 36.89 ± 6.93 µM and 17.72 ± 5.26 µM at 180 minutes (figure 4.17). One conjugate had an earlier retention time than 3'-O-methyl-EC and one eluted later.

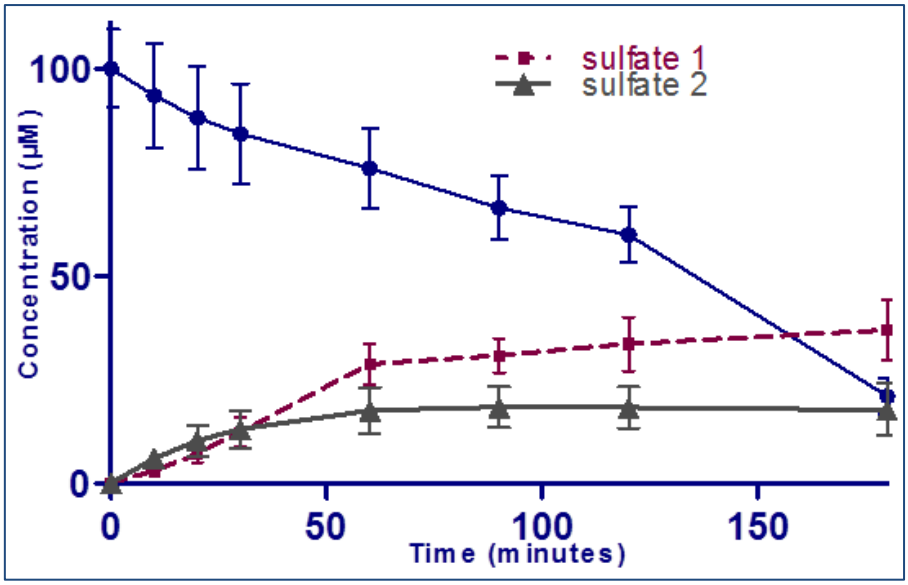

Figure 4.17 – 3'-O-Methyl-EC sulfate formation in human liver microsomes. Values are expressed as mean ± SD, n=6.

4.4– Discussion

The aim of this study was to measure metabolite production of catechins using different enzyme fractions, and therefore be able to use these metabolites in subsequent experiments to assess their protective effects against mitochondrial DNA damage. The results from these metabolism experiments showed significant conjugation which would allow the conjugated mixtures to be used in further experiments. Tea catechins have been shown to undergo extensive conjugation in humans, mainly sulfates and glucuronides which are more water soluble molecules. They can also be rapidly methylated via the actions human placenta cytosolic COMT (Zhu et al., 2000, Vaidyanathan and Walle, 2002).

To be able to understand their bioactivities in vivo, it is vital to know the exact bioavailability of polyphenols, and to what extent they are present in free or conjugated forms. These differ greatly, as EC was shown to be fully conjugated in the plasma, whereas EGCG was approximately 10% conjugated in plasma (Ullmann et al., 2003, Donovan et al., 1999).

In all previous studies looking at either *in vitro* or *in vivo* metabolism of catechins; there have been no observed phase I metabolites. Experiments previously in rats, mice, and human *in vitro* systems have yielded only phase II conjugates (Crespy et al., 2004, Feng, 2006, Kuhnle et al., 2000, Lu et al., 2003a, Lu et al., 2003b, Vaidyanathan and Walle, 2002). Therefore in these experiments the focus was on phase II conjugation of catechins.

This study was carried out to determine the extent of green tea catechins metabolism using phase II conjugating enzymes via *in vitro* enzyme assays at varying time-points up to 180 minutes. The fractions used were human liver microsomes, liver S9, liver cytosol, intestinal microsomes, and porcine liver COMT. The catechins and their conjugates were analysed via ULC/MS and ULC-DAD. Under the experimental conditions used, the quality of the fragmentation spectra was not sufficient which made it impossible to identify the flavanol ring

on which conjugation took place.

Methylation

For the first time, the methylation of catechins using porcine liver COMT was reported. Five methylated metabolites were formed in total; two methylated EGCG metabolites, one EGC methyl metabolite, and two methylated EC products. The interesting thing to note here is that they were all formed using porcine liver COMT and none were detected when catechins were incubated with human liver cytosol. In contrast to these results from the liver cytosol, Zhu *et al* showed in placental cytosol from human term placenta that EC was methylated to form two metabolites and EGC formed one methylated metabolite after incubation for 20 minutes (Zhu et al., 2000). In another study using human COMT in liver microsomes, it was shown that EGCG and EGC were efficiently methylated to 4''-O-methyl-EGCG and 4'-O-methyl-EGC respectively (Lu et al., 2003b), which showed that methylation does indeed occur in human hepatic fractions. Although no methyl metabolites of ECG were detected in either human cytosol or porcine COMT, ECG was methylated by human placenta cytosol, rat liver cytosol, and liver homogenates previously (Okushio et al., 1999, Zhu et al., 2000, Zhu et al., 2001). 200μM concentrations of catechins substrates were chosen in this study to potentially detect metabolites via diode array HPLC which may previously not have been detected due to being below limits of detection and quantification.

EGC produced one methylated metabolite after 180 minutes which was 47.3% of the parent compound. The 3' and 4' sites on the B ring of EGC were revealed as the possible sites of methylation (Lu et al., 2003b). As a HPLC standard for 4'O-meEGC was available and it did not correspond with the retention time of the methyl metabolite produced in the experiments, it was concluded that it was 3'-O-methylEGC.
These findings agree with the results reported previously using rat liver COMT that EGC methylation was faster than EGCG (Zhu et al., 2001, Zhu et al., 2000). However, our experimental results disagree with the data by

Lu *et al* which showed that EGCG had a higher efficiency for methylation than EGC (Lu et al., 2003b). Another difference observed was the lack of any di-methylated EGCG. This was due to EGCG being present at high concentrations of 200μM, whereas the study by Lu *et al* showed that 4'-4''-diMethylEGCG was the predominant metabolite formed at concentrations less than 1μM. The mono-methylated EGCG was the major metabolite at concentrations higher than 3μM EGCG which was the case in our experiments. This also correlates well with in vivo data in rats which showed that 4''-MeEGCG was the highest product excreted in the bile when the rats were given 100mg of EGCG (Kida et al., 2000).
ECG was not methylated in our experiments by either the porcine COMT or the liver cytosol. However, in previous studies ECG and EC were shown to be methylated by rat liver homogenates/cytosol, human placental cytosol, and isolated preparations of rat intestine (Zhu et al., 2001, Zhu et al., 2000, Okushio et al., 1999, Kuhnle et al., 2000). The lack of ECG methyl metabolites could not be explained under our experimental conditions. Two EC methylated metabolites were formed after 180 minutes. EC remained stable in these conditions for more than 180 minutes but the metabolites formed accounted for approximately 11.3% after this time, indicating that EC and its methylated metabolites could have acted as inhibitors of porcine COMT mediated methylation. Further studies are needed to assess the inhibition, if any, of EC and it methyl ethers against COMT especially in human systems.

Glucuronidation and sulfation

All catechins were incubated with UGT cofactors and liver/intestinal microsomes for up to 180 minutes to measure the amounts of glucuronides produced. EGCG was glucuronidated in liver microsomes and s9 fraction to form two glucuronide conjugates in each of the incubations. However in intestinal incubations there were three conjugates present, this could be explained by the presence of an intestine-specific UGT (1A8) acting on a different site of EGCG.
In these experiments a possible hydroxylated glucuronide (m/z 649.1069) conjugate of EGCG was also detected. The exact structural conformation

is yet to be determined. This conjugate was detected in both liver and intestine fractions. However, due to an exact match in the retention time with the glucuronide and no MS/MS conformation, there is not strong enough evidence for the identification of this metabolite here. From the three fractions tested, liver s9 fractions yielded the most metabolism of EGCG. This difference is most likely due to the different expression of some UGT isoforms in the two tissues. EGCG was glucuronidated in liver microsomes, s9, and intestine approximately 24.9%, 42.4%, and 27.5% respectively. This was much higher than EGC which was 4.9%, 7.6%, and 6.2% respectively. This was in agreement to a previous study which showed that UGT mediated Glucuronidation of EGCG was much higher than EGC (Lu et al., 2003).

Crespy *et al* showed that rat microsomes almost completely glucuronidated quercetin, whereas EGCG (7.5 %) and ECG (12.2 %) were glucuronidated much less efficiently (Crespy et al., 2004). This is in stark contrast to our results using human phase II enzyme fractions, where EGCG was glucuronidated between 24.9 and 42.4%, and ECG was comprehensively glucuronidated between 86.5 - 93.6% in liver fractions, and 82.5% in intestinal microsomes. These gallated flavonols are the ones previously reported to be approximately 90% unconjugated in plasma after consumption in humans (Ullmann et al., 2003, Lee et al., 1995, Kotani et al., 2003), which cannot explain the extent of the metabolism observed in our experiments. The action of *in vivo* β-glucuronidase could explain this phenomenon and deconjugate the glucuronides of EGCG and ECG to their free forms, as they are mainly detected in plasma.
A study looking at the glucuronidation and sulfation of EC observed that EC was glucuronidated to two products in rat microsomes, but was not glucuronidated by liver or intestinal human microsomes. It was also shown that EC was sulfated efficiently in human liver and intestine cytosol. They concluded that in the absence of any glucuronides, that sulfation was the major metabolic pathway of EC in human intestine and liver (Vaidyanathan and Walle, 2002). Based on relative quantification to their parent compounds, only trace amounts of sulfate conjugates were observed in

the liver cytosol, and approximately 7.2 % sulfates in liver s9 fractions. The glucuronides produced were also much more pronounced, 19.26 % in liver microsomes, 14.02 % in s9 fractions, and 16.6 % in intestine microsomes. Here it was also reported for the first time, the time-dependant sulfation and glucuronidation of three methylated catechin metabolites; 4'-O-Methyl-EGCG, 4'-O-methyl-EGC, and 3'-O-Methyl-EC.
With only trace amounts of a 4'-O-Methyl-EGCG-sulfate (m/z 551.0464), and the production of five glucuronides (including two di-glucuronides observed for the first time), it was clear that for 4'-O-Methyl-EGCG glucuronidation is also the main metabolism pathway. And similarly, 4'-O-methyl-EGC produced two sulfates in liver cytosol, and reached a combined 47.5%. In liver microsomes, one glucuronide accounted for 91% of 4'-O-methyl-EGC, which showed that glucuronidation was also the major pathway for this metabolite.
However, only 4.08 % of 3'-O-Methyl-EC was glucuronidated after 60 minutes, whereas sulfation of 3'-O-Methyl-EC formed two sulfate conjugates accounting for 54.6 % of 3'-O-Methyl-EC at 180 minutes. Trace amounts of a sulphate cysteine metabolite were also detected for the first time. So in contrast to methylated EGC and EGCG, methylated EC was predominantly shown to be conjugated via the sulfate pathway. The extent of metabolism of these major catechins in our experiments further increases the likelihood that some, if not many, of the in vivo bioactivities of green tea catechins may in fact be wielded by their methylated or sulfate/glucuronide counterparts.

From what was observed in terms of formation of glucuronides in liver and intestine, it would also be important to see the amounts of methyl-glucuronides, methyl-sulfates, and if any sulfate-glucuronides are formed if the enzymes and essential cofactors are pooled together (or just use an S9 fraction to see if there any sulfated glucuronides formed).
Further studies are needed and should incorporate NMR data to elucidate exact structures of the catechin metabolites, and possible dimer/trimers formed. The metabolites formed in our studies show the breadth of phase II metabolism in human intestine and liver, and should

be taken into account in future studies which look at the effects of tea consumption on health effects in humans. The production or availability of metabolite standards should also greatly help with exact quantifications of metabolites produced. Future studies should consider looking at the combined pharmacological activities of these metabolites exactly in the amounts they appear in human plasma which will give a better indication of their effects *in vivo*. The concentrations of the metabolites used in future studies should remain physiologically relevant which will aid in clearing up any uncertainties which stem from using pharmacological concentrations in many *in vitro* studies.

4.5 – References

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5- Protective effects of green tea catechins and their metabolites against mitochondrial DNA damage in intestinal cell lines

Abstract

Background: Mitochondria are the cells main source of ROS production via the electron transport chain. Mitochondrial DNA is close to this site, which makes it susceptible to damage via oxidative stress. MtDNA also possesses less efficient DNA repair machinery than nuclear DNA, and lacks protective histones. It was hypothesised that physiological concentrations of green tea catechins and their metabolites could protect mtDNA against H_2O_2 induced damage in intestinal cells. **Methods:** TC7 and Caco-2 cells were incubated with 50 – 25 µM catechins initially. Thereafter, physiological concentrations of catechins and metabolites were incubated for 4 hours with intestinal cells before the cells were stressed for 24 hours with H_2O_2. Cellular DNA was extracted and analysed via RT-PCR to measure the difference in damage of a long fragment vs a short fragment. Results were compared to a H_2O_2 treated control. **Results:** Higher concentrations of catechins > 25 µM did not protect against mtDNA damage in intestinal cells. When concentrations were reduced to < 5 µM, significant protection against mtDNA damage was observed with all four catechins. Surprisingly, significant effects were also seen at concentrations less than 1 µM. Metabolites of catechins had less bioactivity compared to their parent compounds when protecting against mtDNA damage. **Conclusions:** These results show potent effects of catechins and their metabolites in tissues with high exposure to them after ingestion. Further studies are needed to determine exact mechanisms of action, and potential role of other flavonoids in protecting mtDNA from oxidative damage.

5.1– Introduction

Mitochondria are the predominant cellular organelles for producing ATP and regulation of cellular metabolism, and are therefore referred to as the 'powerhouse' of the cell. They are located near the endoplasmic reticulum, and play roles in apoptosis, lipid biosynthesis, and protein production (Raturi and Simmen, 2013, Riemer et al., 2009, Sinha et al., 2013, Vance, 1990). Mitochondria account for approximately 90 % consumption of the oxygen used by aerobic cells, and ~1-2 % of this oxygen forms reactive oxygen species (ROS) which can subjugate intracellular antioxidant defence systems (Boveris and Chance, 1973). These ROS molecules can cause oxidative damage to the mitochondrial DNA (mtDNA), lipids, and proteins as they are continuously produced in the mitochondrial respiratory chain (Chance et al., 1979). This can lead to increased accumulation of intracellular damage to these macromolecules, along with a decreased antioxidative capacity, and therefore can play a major role in the ageing process.

In contrast to other macromolecules, point mutations and deletions can occur when mtDNA is damaged. These can accumulate and transmit through replication, and therefore deteriorate the integrity of the DNA over the course of multiple replications (Fleming et al., 1982, Miquel et al., 1983). This characteristic is what has made mtDNA damage a core discussion of ageing.

Thirty-seven genes, including 13 polypeptides (all components of the oxidative phosphorylation system), 22 tRNAs, and 2 rRNAs are encoded in mtDNA molecules (Filosto et al., 2011, Mancuso et al., 2009). As these genes code for various mitochondrial proteins and many subunits of the electron transport chain (ETC), mtDNA must remain intact and free from mutations and damage to ensure the normal functioning of the mitochondria (Bohr, 2002). Oxidative phosphorylation (OXPHOS) deficiency and mitochondrial diseases can occur if proper ETC function is not maintained, via the increase in ROS production (Melov et al., 1998, Wallace, 2002).

MtDNA has been reported to be 10- 20- fold more susceptible to damage when compared to nuclear DNA, with the damage being more extensive and lingering for a longer period of time (Stowe and Camara, 2009, Yakes and Van Houten, 1997). This increased susceptibility to damage of mtDNA is attributed to its close proximity to the ROS-rich location of the ETC, and its lack of protective histones which are prevalent in nuclear DNA. MtDNA also has an increased chance of mutations in coding regions owing to the fact that there very few noncoding regions (Anderson et al., 1981, Santos et al., 2012).

Polyphenols and mitochondria

Several epidemiological studies have suggested that dietary polyphenols can reduce the risk of various chronic conditions such as neurodegenerative diseases, some cancers, and cardiovascular disease (Checkoway et al., 2002, Kuriyama et al., 2006). After treatment with micromolar concentrations of Quercetin (10 – 50 μM), caco-2 cells were found to be protected against oxidative stress (Carrasco-Pozo et al., 2012), and accumulate in mitochondria (Fiorani et al., 2010). Indomethacin induced mitochondrial dysfunction was also significantly reduced in caco-2 cells by resveratrol (0.1 mg/ml) and rutin (1 mg/ml) (Carrasco-Pozo et al., 2012). Using C2C12 and mice skeletal myotubes, it was shown that physiological concentrations of resveratrol had a positive effect on mitochondrial function, whereas higher concentrations (100 μM) led to the loss of mitochondrial membrane potential (Higashida et al., 2013). Resveratrol was also shown to improve mitochondrial activity and biogenesis in other studies (Murase et al., 2009, Hart et al., 2013, Um et al., 2010, Menzies et al., 2013). Resveratrol was shown to decrease the production of superoxide anions, and total ROS levels particularly in mitochondria. It appeared to achieve this by inhibiting lipid peroxidation, inducing expression of radical scavenging enzymes such as manganese containing superoxide dismutase, and replenished glutathione levels thereby increasing the level of antioxidant capacity (Leonard et al., 2003, Li et al., 2013). A previous study found that physiological concentrations of EGCG protected against ROS-induced

chromosomal damage in WIL2-NS cells (Sugisawa and Umegaki, 2002). In view of the importance of maintaining mitochondrial integrity, and prevalence of consumption of green tea, it was the aim of the present study to determine the in vitro protective effects of green tea catechins and their metabolites against H_2O_2 induced mitochondrial DNA damage in human intestinal cell lines. The protective effect of green tea catechins against mtDNA damage in intestinal cells has not been explored previously. It was hypothesised that catechins would reduce the H_2O_2 induced mtDNA damage in intestinal cells as these cells are directly exposed to catechins after ingestion of green tea.

5.2– Materials and methods

5.2.1 - Chemicals and reagents

The following were purchased from Sigma Aldrich (Gillingham, UK): Epigallocatechin-3-gallate (EGCG); Epigallocatechin (EGC); Epicatchin-3-gallate (ECG); Epicatechin (Fleming et al.); Ascorbic acid; and Hydrogen peroxide. 3'-O-methyl Epicatechin (Me-EC), 4'-O-methyl Epigallocatechin (Me-EGC), and 4'-O-methyl Epigallocatechin gallate (Me-EGCG) were purchased from Mitsui Norin Co. Ltd (Shizuoka, Japan). Methylated, glucuronidated, and sulfated metabolite mixtures were obtained from enzyme assays (detailed in chapter 4). The following were acquired from Life Technologies (Invitrogen): Dulbecco's modified Eagle's medium (DMEM); foetal Bovine serum (FBS); Penicillin/Streptomycin (1:1); Trypsin-EDTA; non-essential amino acids (NEAA); and L-Glutamine. SYBR-green was obtained from Applied Biosystems (Life Technologies, Netherlands). Primers for Mitochondrial DNA fragments were purchased from Eurofins Genomics (Ebersberg, Germany). The Genomic DNA purification kit was purchased from ThermoScientific (Leicestershire, UK).

5.2.2– Cell culture

Caco-2 cells (human colon adenocarcinoma) obtained from ATCC and Caco-2/TC7 clones were grown routinely in Dulbecco's modified Eagle's medium DMEM, supplemented with 10% Foetal bovine serum (FBS), 2mM Glutamine, 1% pen/strep, and 1% non-essential amino acids. All cells were cultured and maintained in 75cm^2 flasks, with the environment controlled at 37^{o}C and 5% CO_2. The cell medium was changed every 2 days (3 times per week), and cells were sub-cultured at 80% confluence. For the measurement of mitochondrial DNA (mtDNA) damage; 12-well plates were used and were incubated with Catechins at varying concentrations. Cells were seeded onto 12-well plates at 5 x 10^3 cells per well. Ascorbic acid was co-incubated at 5µM. Experiments were carried out 4 days post-seeding when cells were 100% confluent. Caco-2 cells at passage 6-19 were used, and TC7 cells were used at passage 68-79.

5.2.3– MtDNA damage assessment

Both Caco-2 and TC7 cells were cultured in 12-well plates to 100% confluence. They were then washed twice with warm PBS, and incubated with different concentrations of Catechins and metabolites for varying lengths of time. In the case of metabolite mixtures from enzyme assays the same concentration of parent compound was added along with the metabolites, to account for the effect of the remaining parent compound in the enzyme assays at the termination point. Catechins and metabolites were removed by washing the cells again twice with PBS, and then inducing mitochondrial DNA (mtDNA) damage by chronic exposure of the cells to 500 µM H_2O_2 for 24 hours (Caco-2 cells) and 500 µM H_2O_2 for 48 hours (TC7 cells). H_2O_2 concentration and length of exposure was determined via its ability to induce a statistically significant amount of mtDNA damage as well as keeping cell viability >80 %, which was different to H400 cells. These were measured with various dose-response experiments and MTS assay. The concentrations of H_2O_2 used

were 50, 100, 250, and 500 µM for 16, 24, and 48 hours.
The H_2O_2 was removed and genomic DNA was extracted using a cultured mammalian cells Genomic DNA purification protocol (ThermoScientific). The total assay volumes for rt-PCR was 25 µL, which included 40 ng of extracted DNA, forward and reverse primers, and a SYBR green PCR master mix. The 7500 RT-PCR sequence detection software (version 1.3.1, Applied Biosystems) and Applied Biosystems 7500 RT-PCR machine was used for the assays. Mitochondrial DNA damage was expressed as a C_t value (cycles to threshold) fold difference of a long (972bp) mtDNA fragment in relation to a short (83bp) fragment mtDNA primer. The Ct value is inversely proportional to the quantity of DNA and is measured logarithmically as the amount of DNA is theoretically doubled in every PCR cycle.
The primers used were as follows:
Long fragment (972bp) – AL4_F (forward): 5'-CTGTTCTTTCATGGGGAAGC-3' AS1_R (reverse): 5'-AAAGTGCATACCGCCAAAAG-3'
Short fragment (83bp) – IS1 (forward): 5'-GATTTGGGTACCACCCAAGTATTG-3'
IS2 (reverse): 5'-AATATTCATGGTGGCTGGCAGTA-3'.
The PCR program for the long fragment amplification was as follows: 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 55 seconds. For the short fragment amplification the PCR program was: 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 15 seconds.

5.2.4– Statistical analysis

All data were analysed using GraphPad Prism 5.03 (GraphPad software Inc. La Jolla, California). Values were expressed mean ± SEM (n=3) and represented as a difference in C_t values in comparison to untreated controls. A one-way ANOVA with Newman-Keuls multiple comparison tests were carried out to measure statistical significance. Differences were considered significant if *p* values were <0.05.

5.3 – Results

– TC7 cells

The concentration of H_2O_2 and length of exposure which induced significant mtDNA damage and kept cell viability ≥ 80% was determined to be 500 μM for 48 hours. As the H_2O_2 was prone to degradation in cell culture, it was replenished at 24 hours with fresh medium and H_2O_2.
In all experiments, Tiron offered between 88.7 and 96.8 % protection against mtDNA damage. 2mM was used as it had previously been shown to be almost completely protective against mtDNA damage, and in our experiments there was also no significant reduction in cell viability at this concentration.
Catechins added at 50 and 25 μM before exposure to H_2O_2 did not protect against mtDNA damage, and in some cases increased the amount of mtDNA damage slightly (Figure 5.9). In light of this possible pro-oxidant effect of catechins at these high concentrations, lower concentrations were then used (<10 μM).

Catechins show significant protection against mtDNA damage at physiological concentrations

10 μM, 5 μM, and 1 μM of catechins were first incubated with TC7 cells for 18 hours to determine if there was any protective effect against mtDNA damage. The results are shown in figure 5.1. At 18 hours incubation; EGCG and ECG had significant protection across all three concentrations, whereas EGC was not significantly protective at 1 μM, and EC only conveyed significant protection at 10 μM. EGCG and EC showed a concentration dependent increase in protection at 18 hours of incubation. With ECG and EGC, 5 μM showed greater protection than 10 μM (figure 5.1). With significant protection observed after incubation with catechins at 18 hours, the time-point was reduced to a more physiologically representative 4 hours.

The data of the individual catechins at physiological concentrations at 4 hours are shown in figures 5.2 and 5.3. Tiron was chosen as the control which offered almost complete protection against H_2O_2 induced mtDNA damage as shown in a previous study Oyewole et al. (2014). At incubation of 4 hours with individual catechins, concentrations of 1 µM afforded significant protection against H_2O_2 induced damage; EGCG – 69.2±15.1%, EGC – 51±22.4 %, ECG – 74.26± 22.6 %, and EC – 61.49±19.28 %. At 0.5 µM, EC was the only catechin which according to the statistical Newman-Keuls comparison test did not show a significant protective effect. EGCG, EGC, and ECG at 0.5 µM reduced mtDNA damage by approximately 42.6, 41.6, and 39.7 % respectively (figures 5.2 and 5.3).

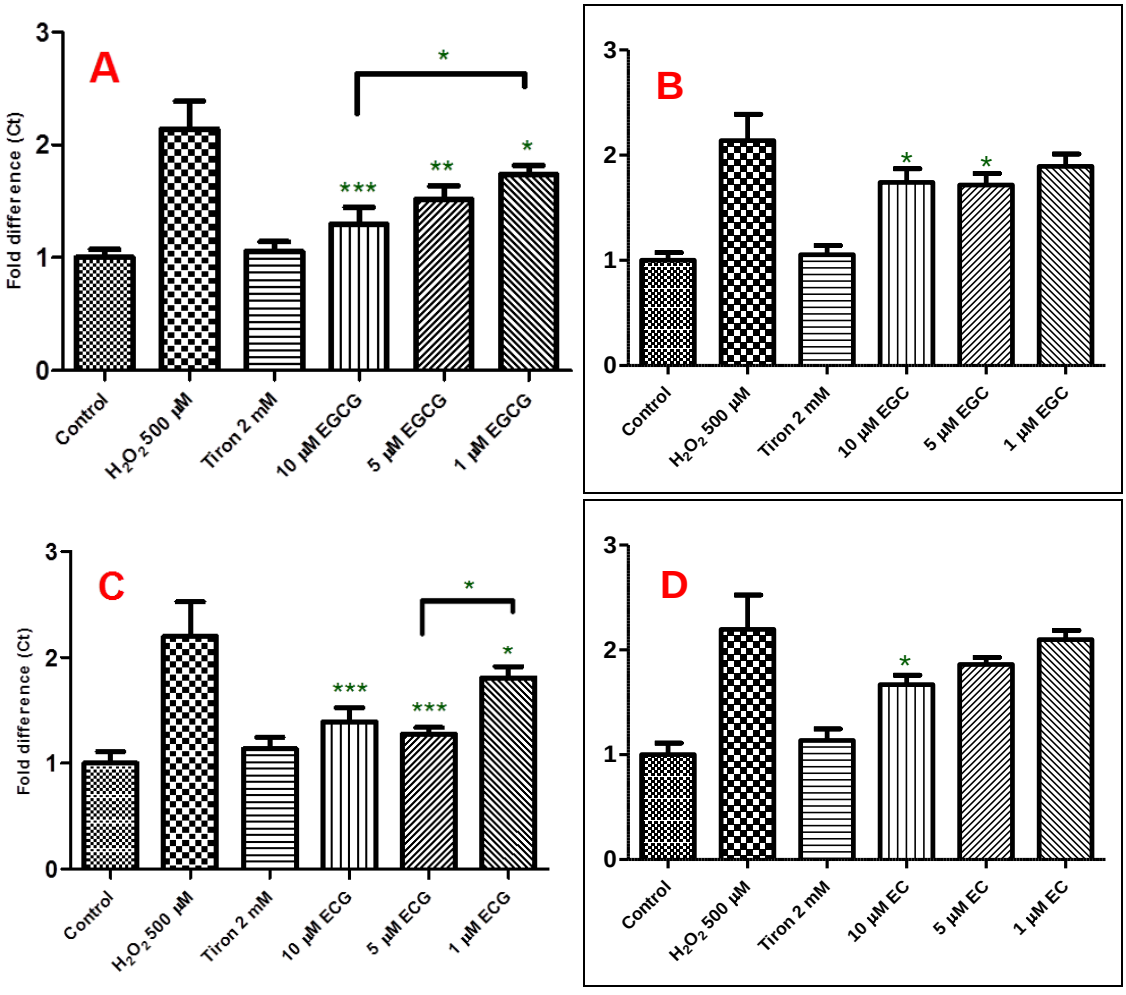

Figure 5.1 – analysis of mtDNA damage after incubation of catechins with TC7 cells for 18 hours. Tiron was used as a positive control in all experiments. Data for EGCG (A), EGC (B), ECG (C), and EC (D) are shown and expressed as the mean value ± SD (n=3). *p<0.5, **p<0.01, ***p<0.001. There were statistically significant differences observed between 10 µM and 1 µM EGCG, and between ECG at 5 µM and 1 µM (*p<0.5).

Metabolites of catechins protect against mtDNA damage in TC7 cells

Two metabolites/ metabolite mixtures were also tested in TC7 cells for their comparative bioactivities in relation to their unconjugated parent compounds. The results are shown in figure 5.4 for these metabolites. 4'O-methyl-EGCG was obtained and used in the mtDNA assay as purified metabolite, whereas the glucuronides for ECG were a mixture of two glucuronides obtained from the UGT enzyme assay (liver s9 fraction). The UGT was stopped at 120 minutes when the approximate concentration of the two combined glucuronides was 90% of the starting parent compound.

In this experiment EGCG and ECG had a protective effect of 68.5 and 71.8 % respectively which was very similar to our previous experiment at 4 hours incubation. 4'O-methyl-EGCG showed a significant 37.8 % reduction in mtDNA damage and the mixture of ECG glucuronides also significantly protected mtDNA by 44.4 %. These results showed that the parent compounds were more bioactive in this experiment as they conferred higher mtDNA protection; in the case of EGCG the difference in bioactivity was significant (* $p < 0.05$). EGCG was 30.7 % more bioactive against mtDNA damage than 4'O-methyl-EGCG, and the ECG glucuronides were 27.4 % less effective than the parent ECG in protecting mtDNA against H_2O_2 induced damage.

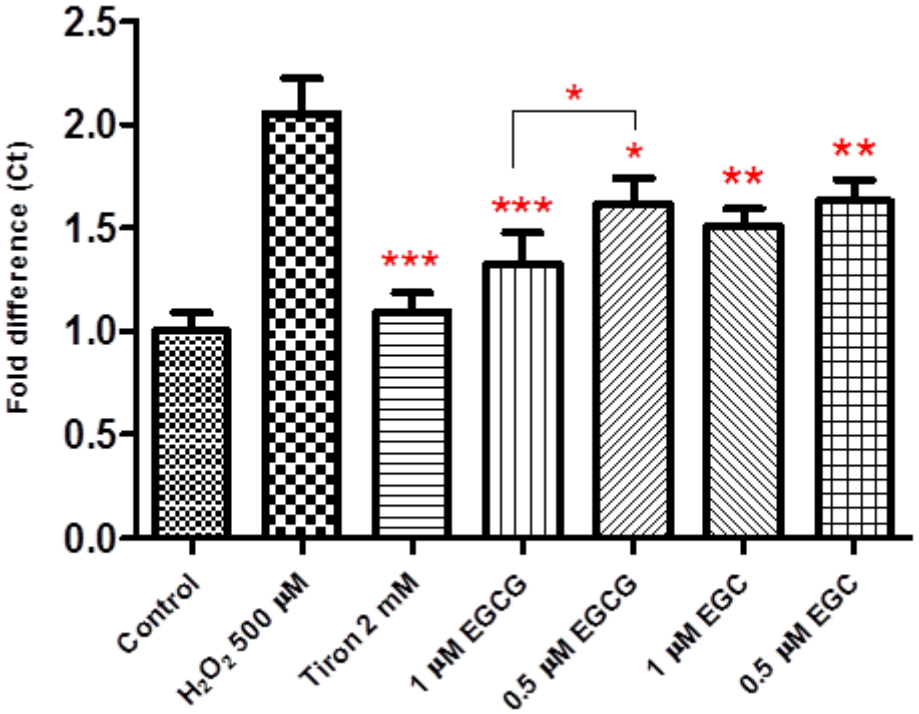

Figure 5.2 – Protective effects of physiological concentrations of EGCG and EGC against mtDNA damage in TC7 cells for 4 hours. Treatments are in comparison to the non-treated H_2O_2 exposed cells (H_2O_2 500 µM). *P <0.05, **P <0.01, ***P <0.001).

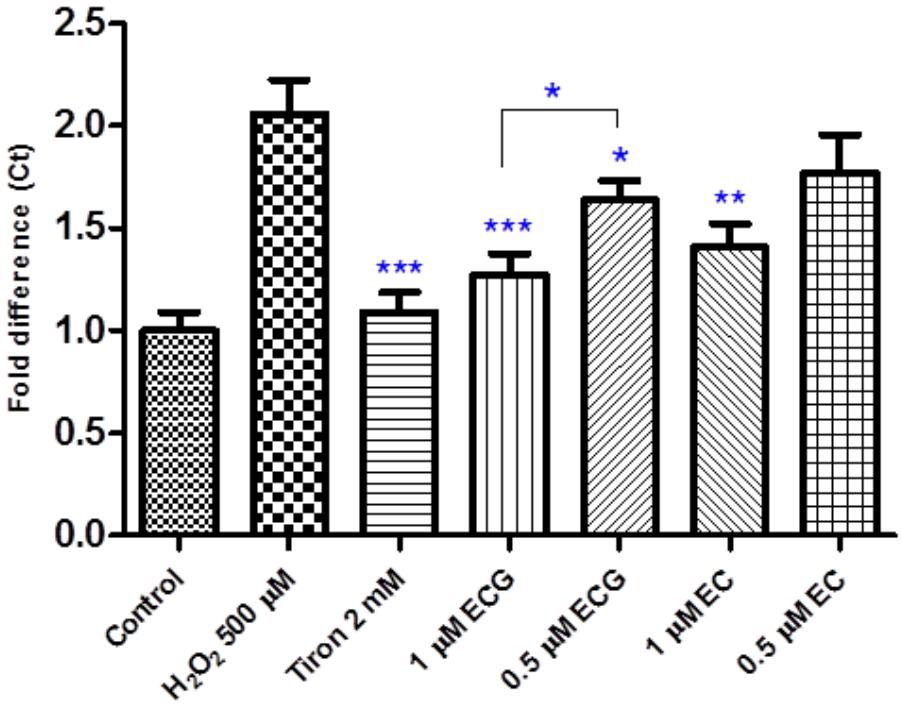

Figure 5.3 – Incubation of physiological concentrations of ECG and EC with TC7 cells for 4 hours. Treatment with 2 mM Tiron offers almost complete protection against mtDNA damage whereas the physiological concentrations of catechins offer less – but significant – protection. Of the four catechins, only 0.5 µM of EC did not seem to offer any significant protection against mtDNA damage

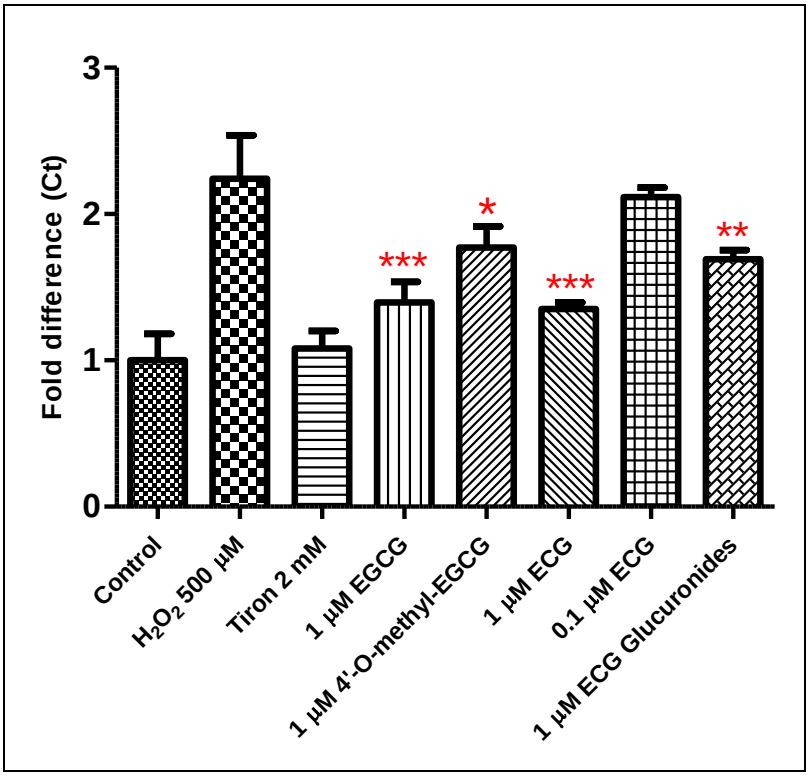

Figure 5.4 – protective effect of catechin parent compounds in comparison to their conjugated counterparts. 0.1 µM of ECG was included to account for approximately the same concentration found in the enzyme mixture containing the two ECG glucuronides. The results show that the metabolites are physiologically bioactive; however, they were significantly less bioactive than their parent counterparts.

The remaining experiments on catechins, their purified metabolites, and conjugated catechins obtained from enzyme assay mixtures for their protective effects against mtDNA damage were carried out in Caco-2 cells. In these experiments 5 µM concentrations for all catechins and conjugates were used as the aim was to only compare the differences in bioactivity between parent catechins and metabolites, as protective effects had already been observed in intestinal TC7 cells. The time of catechin/metabolite incubation remained the same at 4 hours.

EGCG and its metabolites significantly reduce H_2O_2 induced mtDNA damage in caco-2 cells

The results in figure 5.5 show that incubation of EGCG and its metabolites confer statistically significant protection against mtDNA

damage in caco-2 cells, P <0.05 (figure 5.5). All compounds were incubated at the physiological time-point of 4 hours. Interestingly, the methylated form of EGCG showed a 5.7 % increase in protection against mtDNA damage than the parent EGCG at 5 μM although this was not statistically significant. This observation was in stark contrast to the results in TC7 cells at 1 μM where there was a significant 30.7 % reduction in protection of the methylated EGCG compared to the parent compound (EGCG 68.5 % vs 4'O-methyl-EGCG 37.8%).

4'-O-methyl-EGCG was incubated with human liver microsomes to yield 85% glucuronide conjugates after 60 minutes (chapter 4). After 60 minutes approximately 9 % of the original 4'-O-methyl-EGCG was still present. To counter this effect when comparing their mtDNA protective capabilities, 0.53 μM of 4'-O-methyl-EGCG was analysed alongside the glucuronides to account for its effects as it was also present in the enzyme assay mixture at approximately this concentration. This was done for EGCG also which metabolised to glucuronides, where after 180 minutes approximately 3.5 % of EGCG was still remaining so 0.41 μM of EGCG was analysed for comparison with the enzyme mixture. Even at 0.41 μM, EGCG showed a statistically significant protection against mtDNA damage, and the 5 μM EGCG-glucuronide enzyme assay mixture which also contained 0.41 μM EGCG showed a visibly greater protective effect, indicating that the glucuronides had a protective effect against mtDNA damage. The results are shown in figure 5.6. It was also observed that 0.53 μM 4'-O-methyl-EGCG did not have a statistically significant protective effect against mtDNA damage, indicating that the protective effect observed with 5 μM 4'-O-methyl-EGCG-glucuronides was due to the glucuronide conjugates.

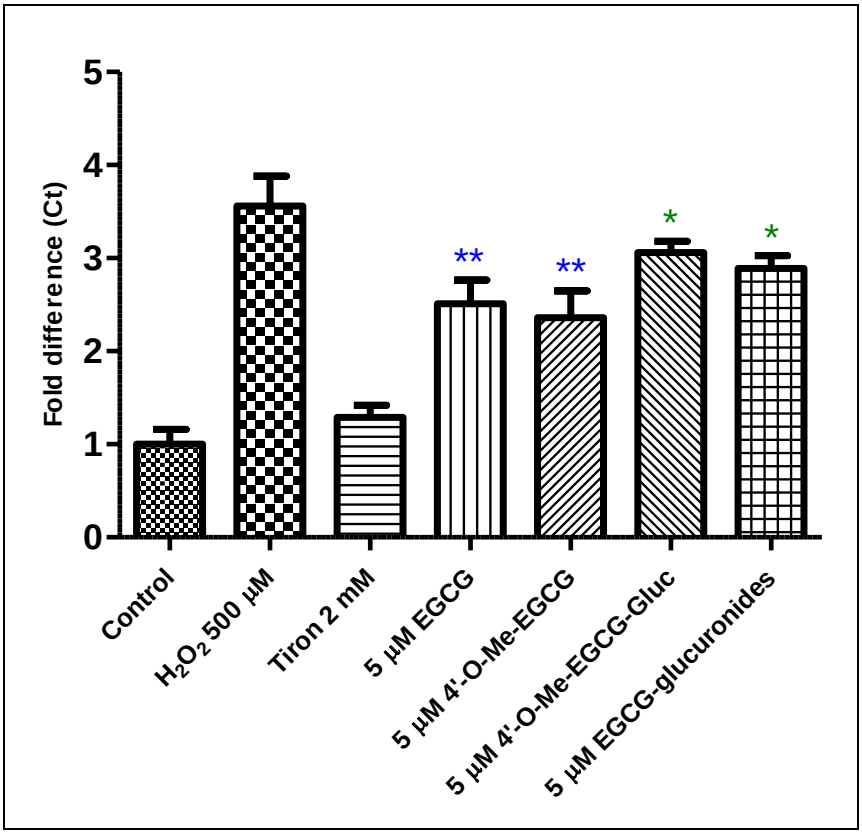

Figure 5.5 – Protective effects of EGCG, its methylated metabolite 4'-O-methyl-EGCG, and their glucuronide conjugates in caco-2 cells. The methylated EGCG was used as a pure compound, whereas the glucuronides were obtained from enzyme assay mixtures. *P <0.05, **P <0.01.

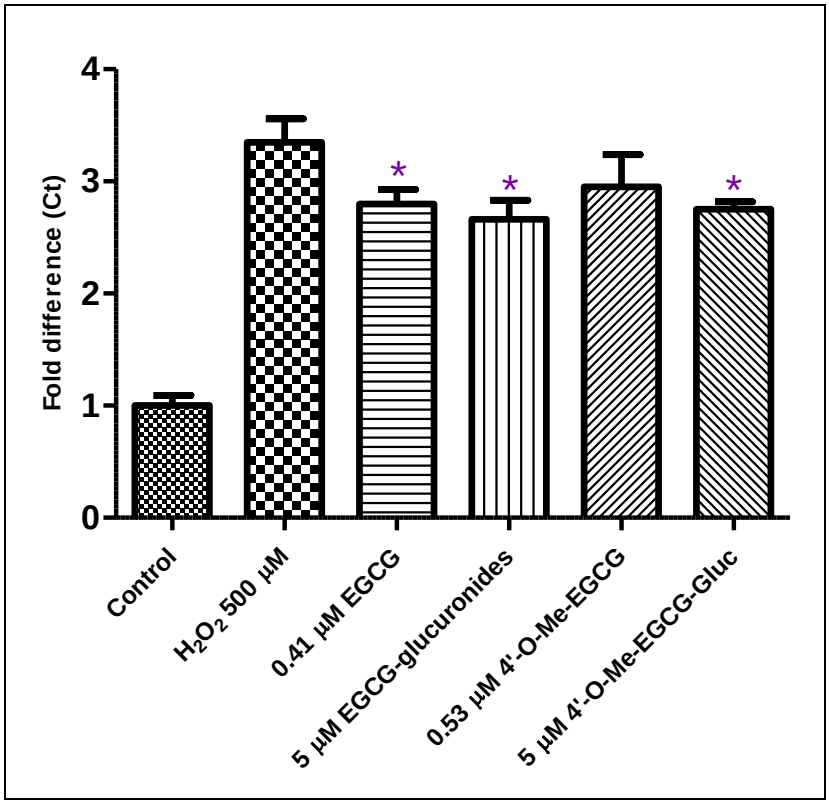

Figure 5.6 – Protective effects of comparative amounts of parent compounds EGCG and its methylated metabolite 4'-O-methyl-EGCG in enzyme assays. 0.41 and 0.53 µM were selected because they were the remaining concentrations of parent compound in the enzyme assays at the selected time points from where the metabolites were used. The results show that the parent compound and metabolites were equally effective but these differences were not statistically significant.

EGC and its metabolites significantly reduce H_2O_2 induced mtDNA damage in caco-2 cells

EGC, 4'-O-methyl-EGC, 4'-O-methyl-EGC-sulfates, and 4'-O-methyl-EGC-glucuronide were added at 5 µM to Caco-2 cells. The sulfates and glucuronide were removed from UGT and SULT enzyme assays carried out previously and the 4'-O-methyl-EGC was the purified compound. The glucuronide assay was continued for 90 minutes to allow for the disappearance of the 'parent' methylated compound, and the SULT assay was carried out for 210 minutes to reduce the methylated EGC to be reduced to trace amounts. The results are shown in figure 5.7.

EGC showed a reduction of mtDNA damage by 45.3 %, compared to 29.5 % for 4'-O-methyl-EGC, 20.8 % for 4'-O-methyl-EGC-glucuronide, and 12.9 % for 4'-O-methyl-EGC-sulfates. Although the methylated metabolite and sulfate/glucuronide conjugates showed significant

mtDNA protection, they were significantly reduced when compared to the parent EGC. The difference is summarised in table 5.1 where the statistically significant treatments are compared to each other.

Newman-Keuls Multiple Comparison Test	***% difference***	***P***
EGC vs 4'-O-Me-EGC-Sulfates	32.4	0.001
EGC vs 4'-O-Me-EGC-Glucuronide	24.5	0.01
EGC vs 4'-O-Me-EGC	15.8	0.05
4'-O-Me-EGC vs 4'-O-Me-EGC-Sulfates	16.6	0.05

Table 5.1 – Direct comparisons of different EGC treatments of Caco-2 cells that were statistically significant. The significance in varying protective effects against mtDNA damage is indicated by the P values. *P <0.05, **P <0.01, ***P <0.001. Data were analysed using one way ANOVA with Newman-Keuls multiple comparison test.

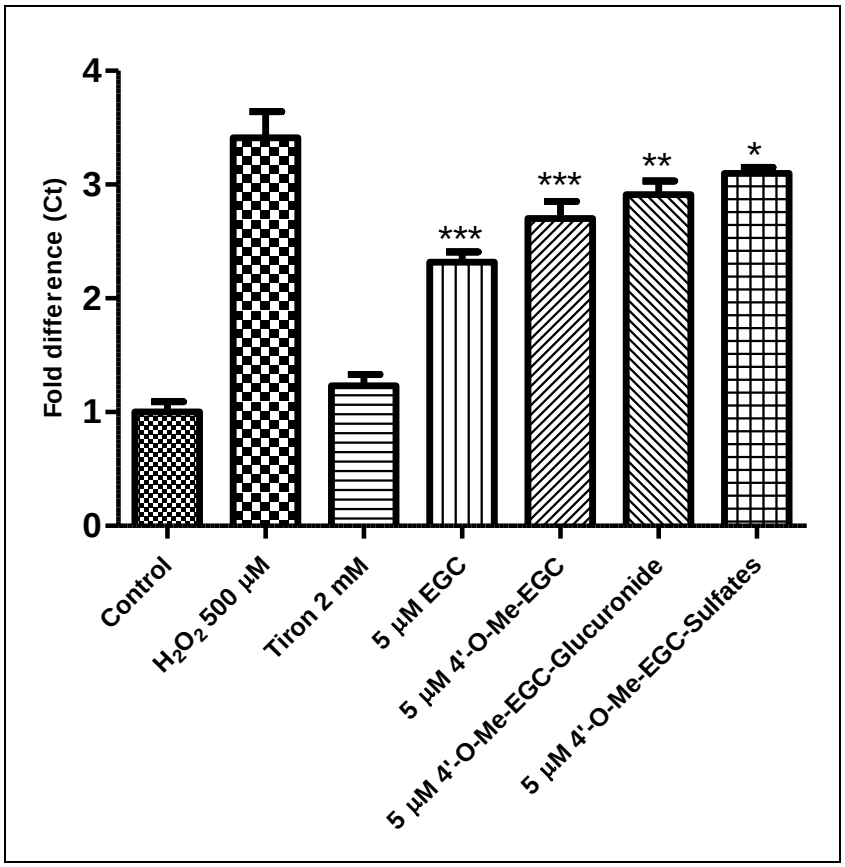

Figure 5.7 – Protective effects of EGC, its methylated metabolite 4'-O-methyl-EGC, and its glucuronide/sulfate conjugates in caco-2 cells. The methylated EGC was used as a pure compound, whereas the glucuronide/sulfates were obtained from enzyme assay mixtures. *P <0.05, **P <0.01, ***P <0.001. n=3 and data are expressed as means ± SD.

The results in figure 5.7 are shown in order of the compounds protective effect. They show that as the extent of conjugation is increased, the protective effect against H_2O_2 induced mtDNA damage is decreased.

EC and its metabolites significantly reduce H_2O_2 induced mtDNA damage in caco-2 cells

EC and its metabolites were incubated with caco-2 cells in the same concentrations as EGC. 3'-O-methyl-EC was metabolised in the SULT assay 210 minutes so that the original metabolite was reduced to approximately 6%. For this reason 0.55 µM of 3'-O-methyl-EC was also tested to compensate for this amount in the sulphate conjugate mixture (Figure 5.8). EC when added to caco-2 cells offered a 53.6 % protective effect, but its metabolites were significantly lower in protecting mtDNA against H_2O_2 induced damage.

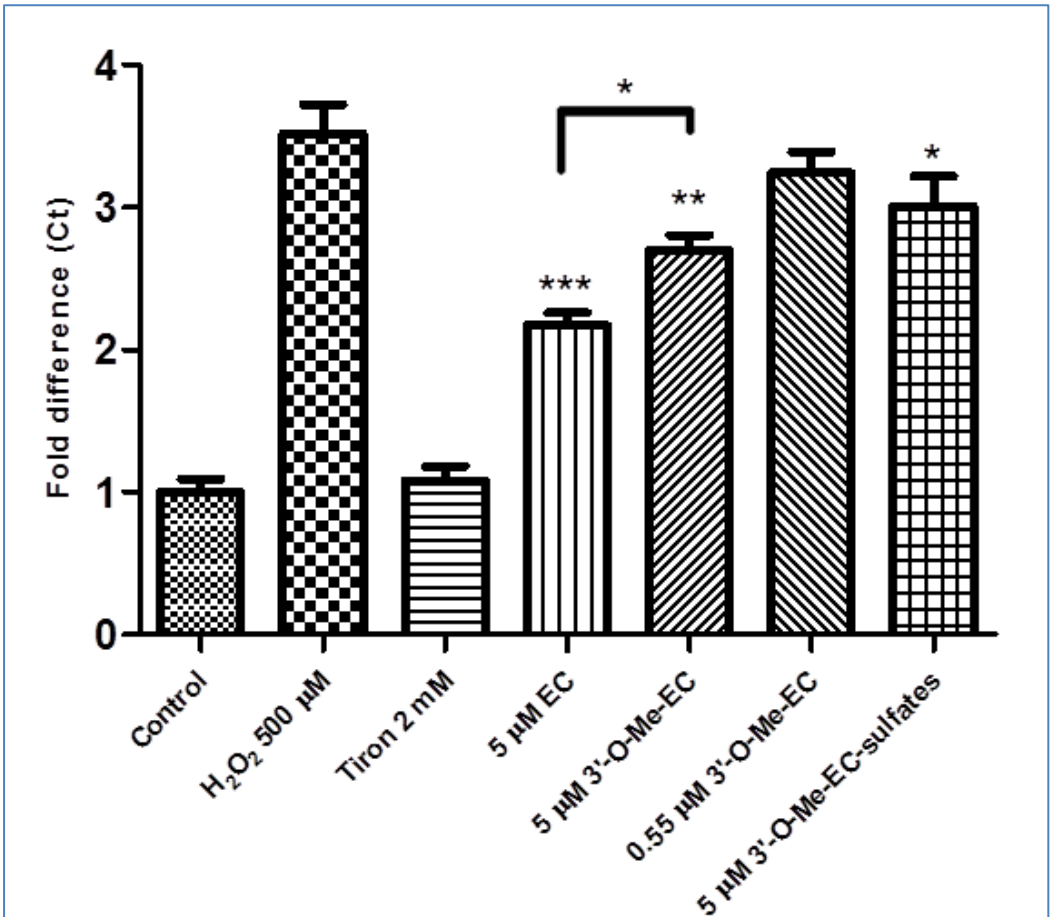

Figure 5.8 – Protective effects of EC, its methylated metabolite 3'-O-methyl-EGC, and its sulfate conjugates in caco-2 cells. The methylated EC was used as a pure compound, whereas the sulfates were obtained from enzyme assay mixtures. 0.55 µM 3'-o-methyl-EC is used here for comparison with the sulfate mixture as this mixture contained approx. the same concentration of 3'-o-methyl-EC. *P <0.05, **P <0.01, ***P <0.001. N=3 and data are expressed as means ± SD.

Although lower than the parent EC, the methylated compound 3'-O-methyl-EC still conferred a significant 32.6 % protection, and the mixture of 3'-O-methyl-EC-sulfates reduced mtDNA damage by 20.3 %. Because the sulfate mixture contained approximately 0.55 µM of 3'-O-methyl-EC, this concentration was also measured for its protective effect. It reduced mtDNA damage by 11.1 % but this was not statistically significant according to the statistical test employed.

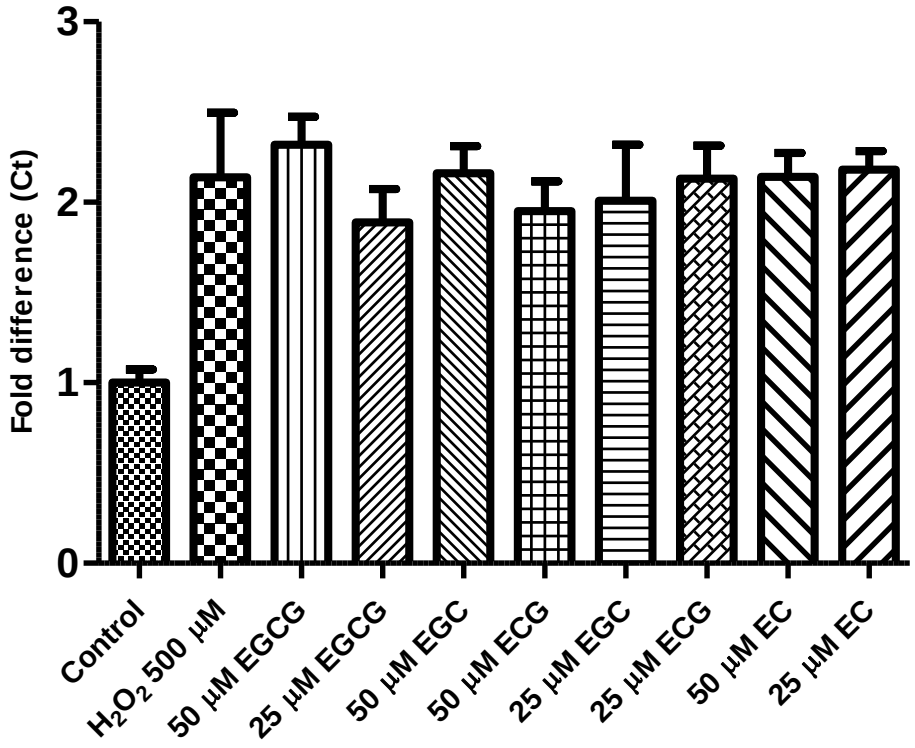

Figure 5.9 – Protective effects of EGCG, EGC, ECG, and EC at 50 and 25 µM in TC7 cells. N=3 and data are expressed as means ± SD. No significant protection was observed at these concentrations. EGCG and EGC at 50 µM had slightly higher amounts of mtDNA damage than the H_2O_2 treated cells.

5.4– **Discussion**

The current *in vitro* studies aimed to examine for the first time, green tea polyphenols and their metabolites for their ability to protect mtDNA against H_2O_2 induced damage in human intestinal Caco-2 and TC7 cell lines. It was shown that physiological concentrations (less than 5 µM) conferred significant protection against mtDNA damage with all four catechins. It was shown that metabolites of catechins had less bioactivity compared to their parent compounds when protecting against mtDNA damage.

The constant formation of ROS by the mitochondrial electron transport chain during the life of the cell leads to an accumulating oxidative stress on mitochondrial function, and is considered a leading factor in cellular ageing. Lipids, proteins, and mtDNA are known to undergo oxidative damage throughout ageing via these oxidative stressors (Shigenaga et al., 1994, Benzi and Moretti, 1995, Sastre et al., 1998). This is thought to be the case when increased ROS production in the mitochondria and faulty antioxidant defence systems results in redox imbalances. Approximately 95 % of the mtDNA molecule is coding regions compared to nuclear DNA which is only 3 %, as mtDNA does not contain introns. Hence any damage to mtDNA is expected to have negative effects on a coding region (Shadel and Clayton, 1997).

In the last couple of decades there has been an increasing interest in the effects of dietary polyphenol consumption and reducing the risk of developing chronic diseases (Kris-Etherton et al., 2002, Visioli et al., 2011). These health effects have often been attributed to the ability of polyphenols to act as efficient ROS quenching bioactives, however there are large differences in the concentrations that are possible in plasma after oral consumption and the concentrations used *in vitro* (Perron and Brumaghim, 2009, Sang et al., 2005). This raises questions regarding the possibility that polyphenols act mainly through ROS scavenging mechanisms *in vivo*. Even so, lower concentrations of polyphenols are generally required to exert their effects via different antioxidant

mechanisms (Fraga et al., 2010) such as; increasing the expression/activity of endogenous antioxidant enzymes, and increasing ROS scavenging enzymes (Lagoa et al., 2011, Tsuji et al., 2013). Recently it been reported that these mechanisms may coincide at different concentrations in different tissues and cellular compartments, and importantly, also in the mitochondria (Schaffer et al., 2012, Visioli et al., 2011). Polyphenols have been shown to control mitochondrial ATP synthesis and its electron transport chain (Carrasco-Pozo et al., 2011), mitochondrial membrane potential (Santos et al., 1998), and induce sirtuins to modulate mitochondrial biogenesis (Chung et al., 2010). Curcumin (8 µM) and resveratrol (100 µM) were shown to protect mtDNA against H_2O_2 induced damage by 16 and 22 % respectively, with 8 and 22 % protection being observed in response to UVA induced mtDNA damage (Oyewole et al., 2014).

The study carried out by Higashida *et al* showed that pharmacological concentrations of resveratrol were cytotoxic in C2C12 cells (in this case greater than 50 µM). Interestingly, they 'adapted' the cells by exposing them intermittently for 6 hours to mild stress inducing concentrations of resveratrol (20 µM), leading to increased mitochondrial biogenesis. This indicated that the mild stress stimulated long term increases in oxidative capacity (Higashida et al., 2013).

Polyphenols undergo significant metabolism after ingestion to their glucuronide and sulfate conjugates, as well as methylated metabolites (Feng, 2006). The reducing hydroxyl groups on the phenol structure are the targets of glucuronidation and sulfation, and this may lead to significant reduction in bioactivity as these are the main phenolic groups to which antioxidant properties are attributed (Piazzon et al., 2012). Sulfation and glucuronide conjugation make molecules more hydrophilic, which can in turn affect their mode/site of activity (Manach et al., 2004). Due to the lack of analytical and purified polyphenol metabolites, there are very few studies which have been able to determine the bioactivities of these conjugates in relation to their parent compounds.

This study helped to determine the *in vitro* bioactivities of purified methylated green tea metabolites, and mixtures of enzyme assay conjugates (glucuronides, sulfates, methyl-glucuronides, and methyl-sulfates) against mtDNA damage in human intestinal cells.

TC7 cells were used to determine concentrations of H_2O_2 deemed to induce significant amounts of mtDNA damage over time-points longer than 18 hours. This was to assess the protective effects of green tea catechins against chronic exposure to damage inducing stressors. Higher concentrations of catechins (50 and 25 µM) produced expected results of slightly increased or unchanged mtDNA damage compared to H_2O_2 treated controls. This is because at higher concentrations of >10 µM, polyphenols can behave as pro-oxidants in vitro and produce extra H_2O_2 (Halliwell, 2008). Those results were consistent with our findings of catechins at concentrations greater than 25 µM. Concentrations this high are not attainable in vivo anyway, so there would be no excess mtDNA damage *in vivo* just by consuming green tea. Of all the catechins/metabolites tested at concentrations below 5 µM, none were found to be cytotoxic or to produce any mtDNA damage.

Tiron conferred almost complete protection (88.7 - 96.8 %) against mtDNA damage in intestine cells. This was in agreement with Oyewole *et al* which showed that tiron was able to permeate the mitochondrial membrane and offer 100 % protection against both H_2O_2 and UVA induced mtDNA damage in cultured skin fibroblasts (Oyewole et al., 2014).

After incubating catechins with intestinal cells for 18 hours to determine if they had any protective effects against mtDNA damage (figure 5.1), the time-point was reduced to 4 hours to give physiologically representative conditions to catechin exposure, and concentrations of catechins were also reduced to 1 and 0.5 µM to represent physiological concentrations. In light of previous studies on green tea catechins and their significant bioactivities (Rosen, 2012), some protection against mtDNA damage was expected. Catechins offered much higher protection than expected (51 – 74.26 %) at physiological concentrations,

and with such a difference in concentration of H_2O_2 (500 µM) and catechins (0.5 – 1 µM) it was unlikely that this protective effect was due solely to the antioxidant properties of catechins. EGCG, EGC, and ECG at 0.5 µM had a protective effect of 42.6, 41.6, and 39.7 % respectively, whereas EC did not show significant protective effects at 0.5 µM. This was unexpected as EC is shown to be more stable in in vitro conditions (Zhu et al., 1997).These results agreed with a study by Sugisawa and Umegaki who showed that physiological concentrations of EGCG prevented ROS induced chromosomal damage in WIL2-NS cells (Sugisawa and Umegaki, 2002). They showed that damage was less in cells treated with 0.3 µM of EGCG than 1 - 10 µM despite the fact that there was no H_2O_2 produced at up to 10 µM EGCG.

The results from our study suggest that catechins have no negative mitochondrial effects in the gastrointestinal tract (intestinal cells in this study, and oral cavity in chapter 3) at physiological concentrations. The mtDNA protective effect of the catechins at physiological concentrations was shown to be occurring inside the cells, as before the cells were exposed to H_2O_2, they were washed twice to remove all extracellular catechins present. However what is worthy of note here is that catechins may have been adsorbed or trapped onto the surface of the cellular membranes and therefore eliciting some protective effects via this action.

The second aim of this study was to ascertain whether or not metabolites of catechins had similar, greater, or reduced capacities to protect mtDNA from damage in relation to their parent compounds.

These results from the metabolite experiments showed that the parent compounds were more bioactive across the board with one exception; EGCG at 1 µM was significantly (30.7 %) more effective than 1 µM 4'O-methyl-EGCG in protecting mtDNA against H_2O_2 induced damage in TC7 cells. However in caco-2 cells at 5 µM concentrations, the parent EGCG showed a 5.7 % decrease in protection when compared to 4'O-methyl-EGCG. This difference between the two concentrations could be explained possibly due to the stability of 1 µM EGCG being lower than 5 µM under cell culture conditions. EGCG and 4'O-methyl-EGCG were

glucuronidated using human liver microsomes and their resulting glucuronide conjugates were also analysed for their protection against mtDNA damage. EGCG glucuronides were significantly effective, yet less than EGCG at protecting mtDNA from damage. Interestingly, the 4'O-methyl-EGCG-glucuronide was less effective than the EGCG-glucuronides. This was unexpected, as usually more conjugation is related to lower bioactivities (Cren-Olive et al., 2003, Duenas et al., 2010, Lu et al., 2003). However this is not always the case, as a study previously also showed that conjugation improved the bioactivity of catechin metabolites in human aortic endothelial cells, inhibiting monocyte adhesion to interleukin 1 whereas the parent catechin did not (Koga and Meydani, 2001). Our results from EGCG glucuronides bioactivity were in agreement with the study of Lu et al, who showed that glucuronides of EGCG and EGC retained siginificant bioactivities and effectively reduced the release of arachidonic acid from HT-29 human colon cancer cells (Lu et al., 2003), however, they used purified metabolites whereas in our study enzyme assay mixtures were used. Because there were small concentrations of parent compounds remaining at the termination time-point of the enzyme assays, a relative proportion of the parent compound was also tested for protection against mtDNA damage (figure 5.6). Here, 0.41 µM of the parent EGCG showed significant reduction in damage, whereas the 4'O-methyl-EGCG at 0.53 µM failed to significantly reduce mtDNA damage, which indicated that the protective effects could indeed be attributed to the conjugates.

The two ECG glucuronides formed in the glucuronide enzyme assay were 27.4 % less effective than the parent ECG in protecting mtDNA against H_2O_2 induced damage. 0.1 µM of parent ECG was also analysed in tandem to ensure the protective effects were not observed due to the ECG remaining at the end point of the ECG glucuronidation assay. Again, even though bioactivity of the ECG-glucuronides was significantly reduced as compared to ECG, the protective effect against mtDNA damage was still visibly significant. The lower activities of the catechin metabolites could be explained by both their reduced antioxidant capacity, and interactions with cell membrane transporters which efflux conjugated flavonoids out of

the cell more than unconjugated flavonoids, therefore reducing the intracellular accumulation (Williamson et al., 2007). EGC and its metabolites also supported the theory that conjugation reduces bioactivity. EGC showed a reduction of mtDNA damage by 45.3 %, compared to 29.5 % for 4'-O-methyl-EGC, 20.75 % for 4'-O-methyl-EGC-glucuronide, and 12.9 % for 4'-O-methyl-EGC-sulfates. Although all the conjugates significantly reduced mtDNA damage, they were all significantly lower than the parent EGC, indicating reduced absorption into the intestinal cells, or increased efflux via ATP-dependant transporters. This is the first time to our knowledge that the bioactivities of 4'-O-methyl-EGC-glucuronide, 4'-O-methyl-EGC-sulfates, and ECG-glucuronides (mixture of 2) have been reported. However, it must be stressed that these metabolites were not present in the experiments in their purified forms and future studies must elucidate the exact structures of these conjugates, and then purify them to use in the studies. This will enable their exact bioactivities to be determined without any hindrance from other assay components.

Another novel aspect of this study was measuring the bioactivity in terms of protection against mtDNA damage of 3'-O-methyl-EC-sulfates. These were formed after incubation of 3'-O-methyl-EC with liver cytosol (chapter 4) over 210 minutes. 3'-O-methyl-EC showed significant bioactivity, protecting the mtDNA by 32.6 % from damage, which agreed with previous studies of the activity of methylated EC (Duenas et al., 2010, Nakagawa et al., 2007). The mixture of 3'-O-methyl-EC-sulfates reduced mtDNA damage by 20.3 %, which was a 33.3 % reduction in protective capacity when compared to the parent compound. Some cells have the ability to deconjugate metabolites to their parent compounds, which might enhance their absorption and reduce their efflux, which may contribute to the bioactivity of these conjugates.

In conclusion, the results presented in this chapter show that green tea catechins offer greater protection against mtDNA damage than their conjugated counterparts, although the conjugates still retain significant protective activities against mtDNA damage. Conjugation of catechins

generally reduces bioactivity, and this may be due to greater difficulty in entering and accumulating in cells, greater affinity for efflux transporters, and conjugation on the main ring to which the specific bioactivity is attributed to. Dietary catechins also show they have a potential new function – independent of their antioxidant function - of protecting mitochondria. These data together show that even though bioavailability of catechins is low *in vivo*, these catechins and their metabolites in small concentrations could synergistically protect mitochondria from external oxidative stressors.

Of course more detailed studies are needed which would help to elucidate the exact mechanism by which catechins and their metabolites protect mtDNA from H_2O_2 induced damage. As mentioned previously, these experiments would have to use purified metabolites rather than mixtures obtained from enzyme assays.

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Chapter 6 – Inhibition of Green tea catechin glucuronidation by Piperine and Quercetin-3-β-glucoside

Abstract

Background: Green tea catechins have shown increasing health benefits over the last two decades. Their extensive metabolism and low absorption however make their bioavailability very low *in vivo*. New strategies are needed to potentially increase the bioavailability of beneficial flavonoids. piperine and quercetin are known to inhibit intestinal glucuronidation. Iy was hypothesised that both these compounds would be efficient inhibitors of intestinal catechin glucuronidation. **Methods:** Enzyme assays were carried out using human intestinal microsomes as both quercetin and piperine showed no inhibition towards liver microsomes. Assays were run with and without piperine or quercetin. Samples were removed after certain time-points and analysed via ULC/MS. **Results**: Piperine and quercetin both significantly reduced the glucuronidation of EGC and EC. Glucuronidation of EGCG and ECG was also reduced, however was not statistically significant for all individual metabolites. Piperine was a more effective inhibitor than quercetin for the non-gallated catechins, and quercetin was more effective in the case of the gallated catechins. **Conclusions:** This study shows that common dietary compounds which can inhibit certain phase II conjugation pathways may potentially increase the bioavailability of beneficial polyphenols in the diet. Further studies are needed to determine how this effect on metabolism occurs in cell lines and in vivo. Other dietary compounds should also be explored to potentially increase bioavailability of tea catechins.

6.1– Introduction

Green tea catechins have been shown to have possible health benefits in numerous in vitro, animal, and human studies (Henning et al., 2011, Jung et al., 2001, Yang and Wang, 2010). The main catechins attributed to the beneficial effects of green tea are Epigallocatechin (EGC), Epicatechin-gallate (ECG), Epigallocatechin gallate (EGCG) (the most abundant, accounting for up to 50-80 % of catechins), and Epicatechin (EC) (Sang et al., 2011).

The health benefits of green tea catechins are somewhat limited due to low bioavailability, which is due to limited absorption, extensive phase II metabolism, and efflux (Lambert et al., 2007). As the potential benefits of catechins are dependent upon the concentrations circulating in plasma after absorption, strategies which can improve bioavailability are much sought after. These strategies could include reducing efflux by cellular transporters, phase II conjugating enzyme inhibitors, or compounds which can increase cellular uptake of a target polyphenol.

Catechins and other polyphenols, must first pass the intestinal epithelium, which is full of phase II enzyme containing enterocytes (Won et al., 2012). Phase II metabolism occurs extensively in the liver and intestine, with the major pathway usually being glucuronidation in humans (Ritter, 2007, Wu et al., 2011). Dietary constituents and herbs have been shown previously to inhibit intestinal glucuronidation (Li et al., 2012, Mohamed and Frye, 2011). Metabolites of catechins tend to have reduced bioactivities when compared to their parent compounds, but some metabolites have also demonstrated equal bioactivities to their parent counterparts (Cren-Olive et al., 2003, Duenas et al., 2010, Lu et al., 2003).

Other methods of attempting to enhance bioavailability of polyphenols have tried to change the site of absorption to the small intestine, improving metabolic stability, and new methods of delivery (Cao et al., 2013, Nielsen et al., 2006, Walle, 2007, Zhang et al., 2011). An easier, cheaper, or faster method favourably modifying the bioavailability of

catechins would be to consume common dietary compounds or foods that have been shown to inhibit intestinal or liver phase II metabolism, or increase absorption (Monobe et al., 2011). Piperine (figure 6.0) is an alkaloid and is one such compound which has been shown previously to inhibit glucuronidation in rats (Singh et al., 1986), and inhibit intestinal glucuronidation of EGCG and increase its bioavailability in mice (Lambert et al., 2004), however the inhibition of EGC, ECG, and EC glucuronidation was not analysed in this study. Quercetin (figure 6.0) is also a known inhibitor of Glucuronidation, more specifically a mixed-type inhibitor (Mohamed and Frye, 2010) and a common flavonoid in the diet; being present in apples, broccoli, garlic, raw onions, spinach, and red wine (Aherne and O'Brien, 2002).

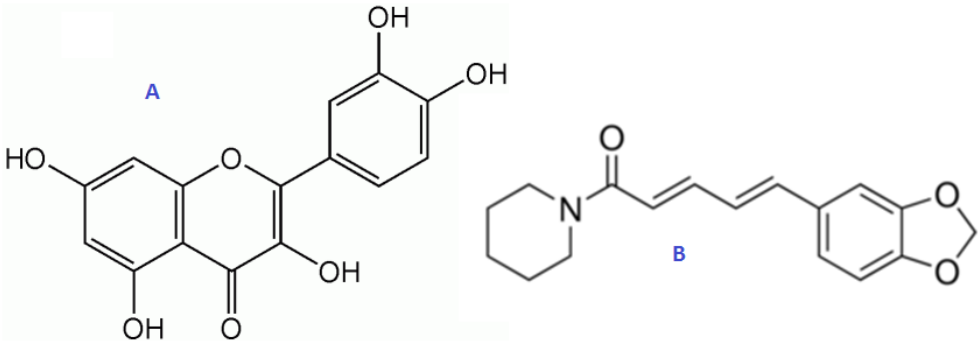

Figure 6.0 – Structures of quercetin (A) and piperine (B). Quercetin acts as a mixed-type inhibitor, and piperine as a non-competitive inhibitor of intestinal glucuronidation

As a common dietary component, and modulator of UGT activity, the aim was to analyse the inhibitory effects of Quercetin on green tea catechin glucuronidation via intestinal and liver microsomes.

The study of Piperine and EGCG did not mention any of the other catechin being analysed for inhibition of glucuronidation. And to our knowledge it has not been reported elsewhere. It was hypothesised that because Piperine inhibited EGCG glucuronidation, it could also likely inhibit glucuronidation of the other three major catechins.

6.2– Materials and methods

6.2.1 – Chemicals and reagents

The following were purchased from Sigma Aldrich (Gillingham, UK): Ascorbic Acid; Catechins (EGCG, EGC, ECG, & EC); Molecular biology grade H_2O; Piperine; Quercetin-3-β-glucoside; and 0.5 Molar Tris-HCl buffer. ULC/MS-grade Acetonitrile; ULC/MS-grade methanol; Acetic acid; and Formic acid were purchased from Biosolve BV (Valkenswaard, the Netherlands). Human liver microsomes (HLM), and human intestinal microsomes (HIM), UGT reaction mix A (25mM UDPGA), UGT reaction mix B (250mM Tris-HCl buffer, 40mM $MgCl_2$, 0.125mg/mL Alamethicin) were acquired from BD biosciences (Swindon, UK). The UGT-Glo™ assay kit was purchased from Promega (Southampton, UK) to measure inhibition of UGT activity.

6.2.2 – UGT-Glo™ assay

The UGT-Glo™ assay was used to measure the inhibitory effects of Quercetin and Piperine on intestinal and liver microsome glucuronidation. Pro-luciferin substrates were used (supplied by the assay) to measure UGT activity via luminescence. Varying concentrations of Piperine and Quercetin-3-β-glucoside (0.5 – 400 µM) were used to determine the IC_{50} values of these compounds to inhibit glucuronidation.

6.2.3 – Intestinal glucuronidation of Catechins and inhibition via Quercetin and Piperine

Glucuronide assays were carried out using the manufacturer's instructions (BD biosciences). Molecular biology grade H_2O, UGT reaction mix A, UGT reaction mix B, 200 µM catechin substrate, the IC50 concentrations of Piperine and Quercetin-3-β-glucoside, and 50µM ascorbic acid were combined in a total volume of 1mL and warmed to

37ºC for 5 minutes. The reactions were initiated using 0.2mg of Human intestinal microsomes HIM. Samples of 100µL were removed at 0, 10, 20, 30, 60, 90, 120, and 180 minutes and added to 100µL of ice-cold methanol containing 1% ascorbic acid and acetic acid. After centrifugation at 10000g for 10 minutes the supernatants were analysed via LC/MS. The assays were repeated twice.

6.2.4 – Data acquisition

Analysis of catechins and their metabolites was carried out using mass spectrometric detection in negative ionisation electrospray mode on an Acquity UPLC system and Synapt-G2 HDMS (Waters, UK). Separation was acquired via an Xbridge C_{18} column (50mm x 4.6mm i.d., 3.5µm) at a flow rate of 0.3mL/min; and column temperature was maintained at 35ºC. The sample injection volume was 3 µL. The sampling cone voltage was set at 35V; desolvation temperature at 350ºC; and source temperature at 100ºC. Leucine Enkephalin (2ng/mL) was used as a reference compound in negative ion mode (m/z 554.2648). The mobile phases were (A) 0.1% Formic acid in water (w/v) and (B) 0.1% Formic acid in acetonitrile. Initial conditions were 3% B for 1 minute, increasing to 18% B at 6 minutes, staying constant at 18% B until 6.5 minutes, back to 3% B at 6.6 minutes, and then stayed at 3% B until 8 minutes. Accurate masses were obtained using Masslynx 4.1 software, with scanning across the spectrum of 50-1000 m/z.

6.2.5 – Data analysis

All data were analysed using GraphPad Prism 5.03 (GraphPad software Inc. La Jolla, California). Values were expressed mean ± SD (n=2) and statistical significance was determined using one-way ANOVA with Tukey's test. Differences were considered significant if *P* values were <0.05.

6.3– Results

The UGT-Glo™ assay was carried out to determine if Piperine and Quercetin had inhibitory effects on glucuronidation in human liver and intestinal microsomes (figure 6.1). The IC_{50} of Piperine inhibition on intestinal glucuronidation was 106.4 µM. This was the concentration used in further inhibition experiments with green tea catechins. There was no inhibition observed with Piperine in liver microsome glucuronidation, even at 500 µM. The IC_{50} of Quercetin-3-β-glucoside inhibition of intestine microsome glucuronidation was 15.2 µM. As seen with Piperine, there was no inhibition of glucuronidation observed in liver microsomes.

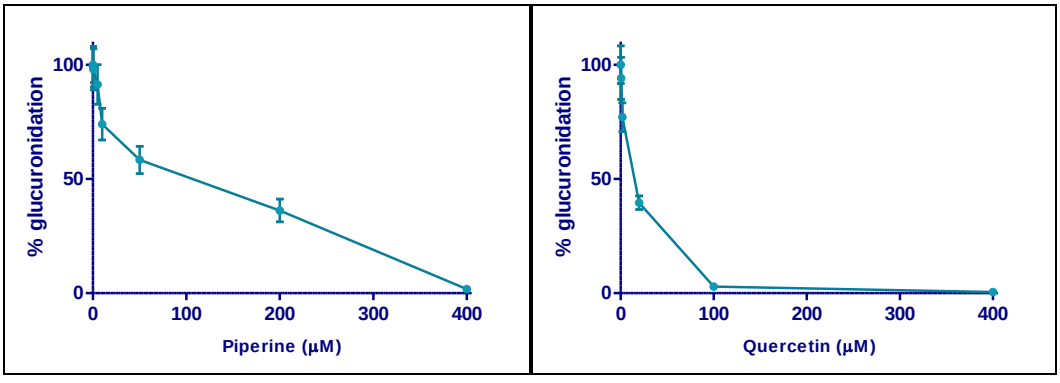

Figure 6.1 – Incubation of various concentrations of Piperine and Quercetin with human intestinal microsomes in the UGT-Glo assay to determine their inhibitory effects against intestinal glucuronidation. The data was analysed using GraphPad prism to obtain IC_{50} values for both compounds; 15.2 µM for Quercetin and 106.4 µM for Piperine. Values are expressed as the mean ± SD (n = 3). Incubation time in the UGT-glo assay was as instructed in the UGT-glo assay protocol (Promega).

Inhibition of EGCG glucuronidation

The results in chapter 4 showed that catechins were efficiently conjugated by UGT enzymes in human liver and intestinal microsomes. For this reason glucuronidation was chosen as the metabolism pathway in the *in vitro* systems to test for inhibition via common food compounds. However, as there was no inhibition observed via liver microsomes, the focus was instead on intestinal inhibition of glucuronide formation.

After incubation of EGCG with intestinal microsomes, three glucuronides were produced (chapter 4, figure 4.4).Piperine was shown to significantly reduce glucuronide conjugation of only one EGCG glucuronide (G5, chapter 4, figure 4.4) when incubated with intestinal microsomes. Figure 6.2 shows the reduction in EGCG glucuronide formation in the presence of 106.4 µM Piperine. Each experiment was repeated in duplicate. Although there were visible differences in the conjugation of EGCG as shown with the individual concentrations of glucuronides, the only significant reduction was in the formation of glucuronide 1 (G5, figure 4.4 in chapter 4) (figure 6.2). Piperine inhibited total glucuronide conjugation by approximately 42.8 %, 48.1 %, 43.7 %, and 27.2 % at 10, 20, 30, and 60 minutes, respectively. Figure 6.3 shows the reduction in EGCG glucuronide formation in the presence of 15.2 µM Quercetin-3-β-glucoside. Glucuronide conjugation was inhibited by 58.9 %, 62.1 %, 61.4 %, and 42.1 % at 10, 20, 30, and 60 minutes respectively.

In the case of EGCG, Quercetin-3-β-glucoside showed a higher inhibitory effect than Piperine on glucuronide formation at all time-points; 16.1 % at 10 minutes, 14 % at 20 minutes, 17.7 % at 30 minutes, and 14.9 % at 60 minutes.

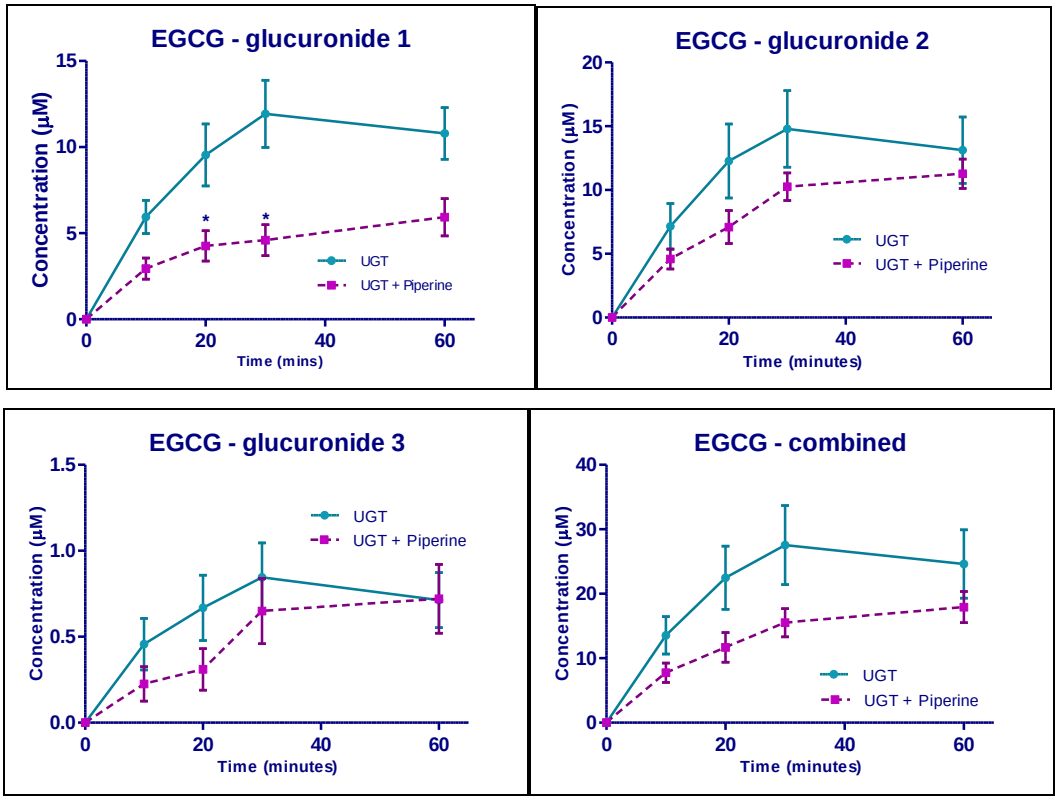

*Figure 6.2 – EGCG glucuronidation with intestinal microsomes in the presence of Piperine. Formation of all three glucuronide conjugates was reduced; however, only the first glucuronide (glucuronide 1 - top left) showed significant inhibition by Piperine at 20 and 30 minutes. The combined glucuronides are also shown but did not show statistically significant inhibition of EGCG glucuronide production (bottom right). Values are expressed as the mean ± SD (n = 2). *P < 0.05.*

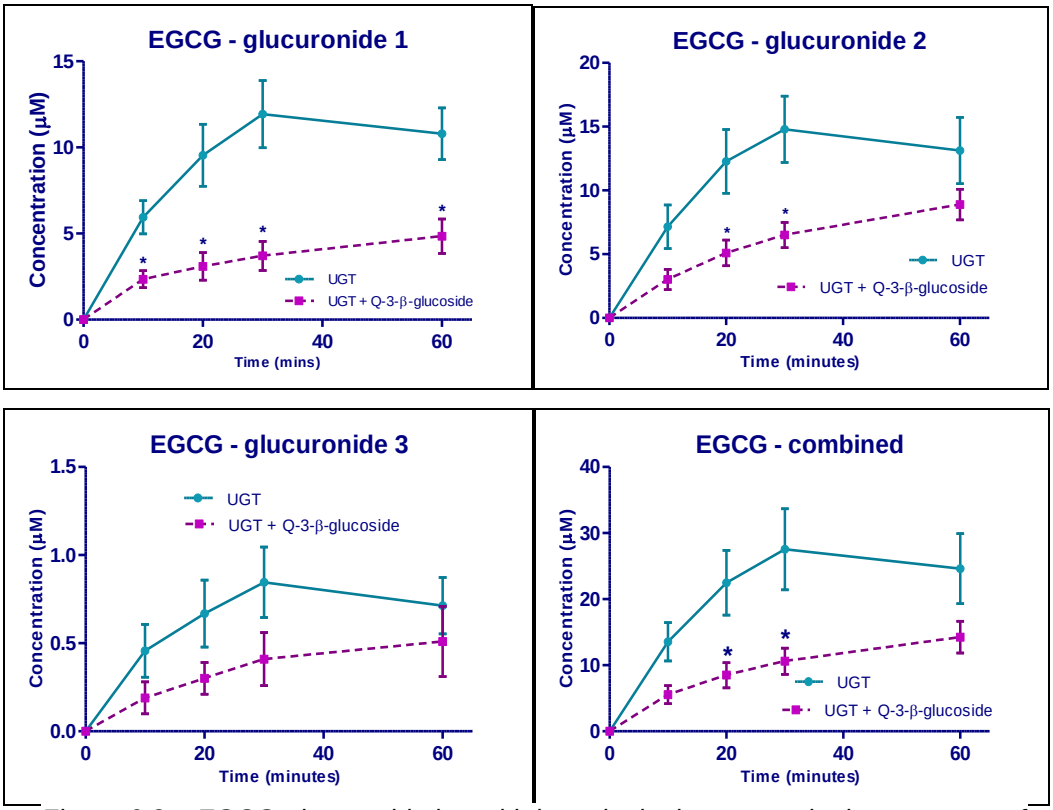

*Figure 6.3 – EGCG glucuronidation with intestinal microsomes in the presence of Quercetin-3-β-glucoside. Formation of all three glucuronide conjugates was reduced; however, only 2 glucuronides (glucuronides 1 and 2 - top) showed significant inhibition at 10, 20, and 30 minutes. The combined glucuronides are also shown and had significantly increased inhibition of EGCG glucuronide production at 20 and 30 minutes (bottom right). Values are expressed as the mean ± SD (n = 2). *P < 0.05.*

Inhibition of EGC glucuronidation

After incubation of EGC with intestinal microsomes, two glucuronides were produced (chapter 4, figure 4.8). Both glucuronides were produced in relatively low amounts compared to ECG and EGCG glucuronides. One glucuronide reached a peak concentration of 5.03 µM, and the second minor glucuronide conjugate reached 1.22 µM at 90 minutes. When EGC was incubated in the presence of Piperine and Quercetin-3-β-glucoside, the formation of the minor glucuronide (G9, figure 4.8) was not affected. The results for the glucuronide (G8) which was inhibited by both compounds are shown in figure 6.4. The inhibitory effects of both Piperine and Quercetin on G8 were similar up to 30 minutes, at which point the difference in inhibition became clearer, Piperine being the superior inhibitor up to 90 minutes. Piperine inhibited EGC-glucuronide 1

(G8) conjugation by 72.1 %, 77.6 %, 75.1 %, 78.9 %, and 76.9 % at 10, 20, 30, 60, and 90 minutes respectively. At the same time points Quercetin inhibited glucuronidation of (G8) EGC by 74.6 %, 75.1 %, 69.9 %, 55.5 %, and 42.4 %.

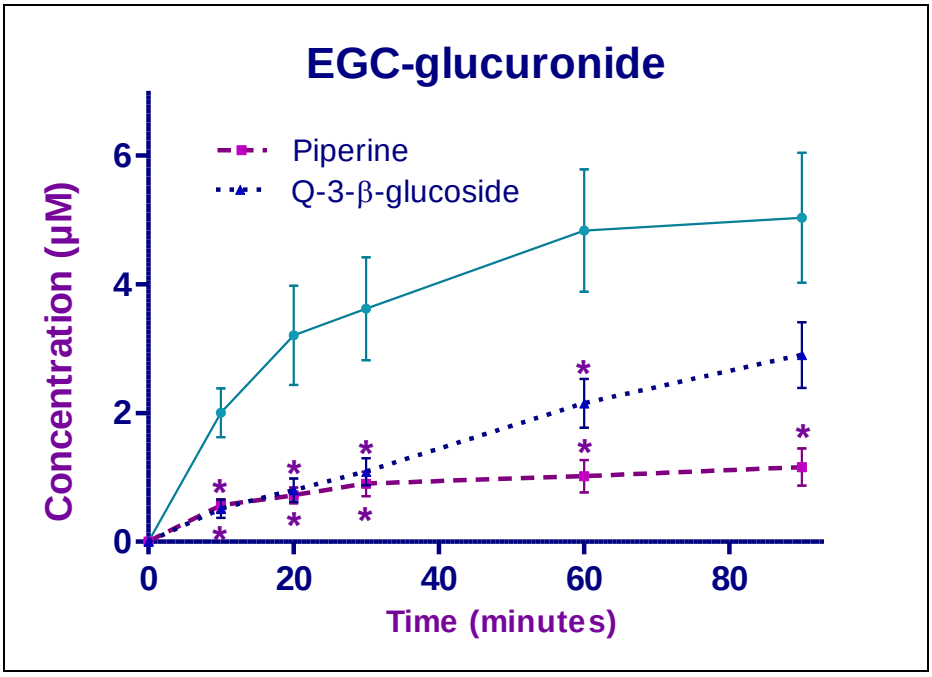

*Figure 6.4 – EGC glucuronidation with intestinal microsomes in the presence of Quercetin-3-β-glucoside and Piperine. Formation of the main glucuronide conjugate was reduced significantly as established via one way ANOVA with Tukey's test; Piperine inhibited the glucuronide formation significantly at all time-points, and Quercetin-3-β-glucoside showed statistically significant inhibition up to 60 minutes. Values are expressed as the mean ± SD (n = 2). *P < 0.05.*

Inhibition of EC glucuronidation

Figure 6.5 shows the results of co-incubation of EC with Piperine and Quercetin. The inhibition followed the same pattern as EGC, in the sense that Piperine was the more efficient inhibitor of EC glucuronidation than Quercetin at their respective IC_{50} concentrations. The results of the glucuronide shown were the major glucuronide (G15, figure 4.16) formed with EC and intestinal microsome incubation. Both Piperine and Quercetin had no inhibitory effect on the conjugation of the second, minor EC glucuronide (G16, figure 4.16). Piperine reduced EC glucuronide formation by statistically significant 78.1, 74.4, 78.2, 73.8,

and 66.4 % at 30, 60, 90, 120, and 180 minutes respectively. With Quercetin, the reduction in glucuronide conjugation was only calculated as significant at 60, 90, and 180 minutes where the inhibition was 51.4 %, 52.6 %, and 46.6 % respectively. Although not deemed significant statistically, there were still distinct reductions in glucuronide formation at 30 and 120 minutes, where the inhibition was calculated as 35.3 % and 44.5 % respectively.

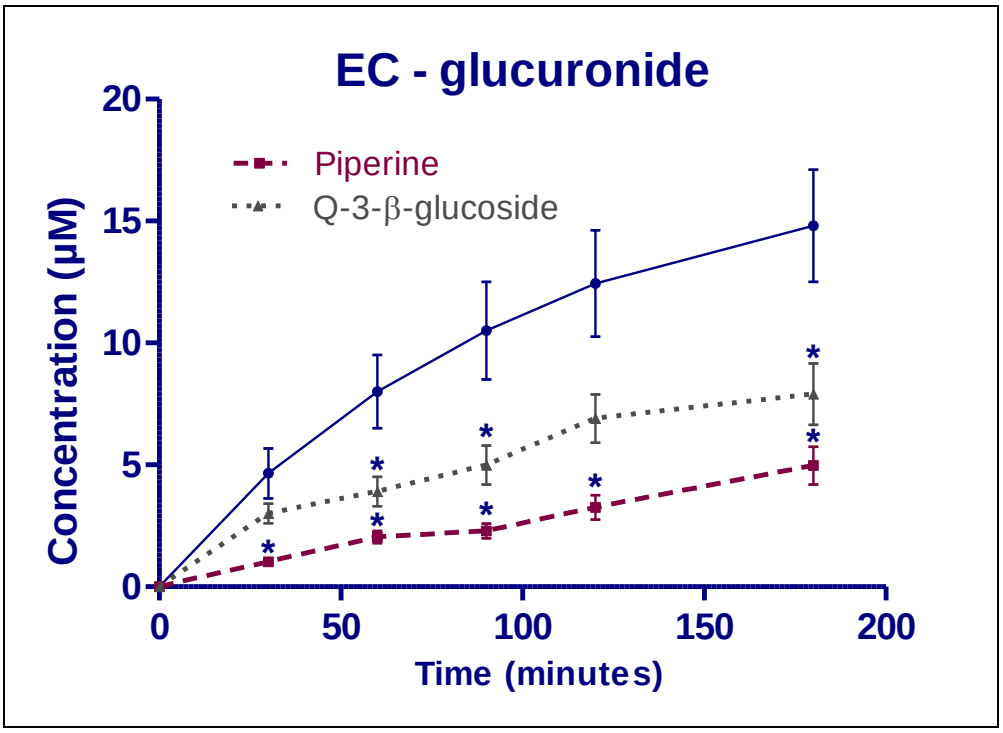

*Figure 6.5 – EC glucuronidation with intestinal microsomes in the presence of Quercetin-3-β-glucoside and Piperine. Formation of the main glucuronide conjugate was reduced significantly as established via one way ANOVA with Tukey's test; Piperine inhibited the glucuronide formation significantly at all time-points, and Quercetin-3-β-glucoside showed statistically significant inhibition at 60, 90, and 180 minutes. Values are expressed as the mean ± SD (n = 2). *P < 0.05.*

Inhibition of ECG glucuronidation

Four glucuronides were produced after incubation of ECG with intestinal microsomes (figure 4.15, chapter 4). The combined concentration of the glucuronides reached approximately 83 % after 180 minutes. The main glucuronide (G11) reached a maximum concentration of 53.34 μM, and the other three metabolites reached 7.2 (G12), 12.3 (G13), and 9.6 μM

(G14) after 180 minutes. Figure 6.6 shows the results of co-incubation of ECG with Piperine and Quercetin. Piperine was shown to be less effective than Quercetin at inhibiting glucuronidation of ECG for the three minor metabolites, however, had a visibly greater impact on glucuronidation for the major ECG glucuronide (glucuronide 4 in figure 6.6, G11 in figure 4.15).

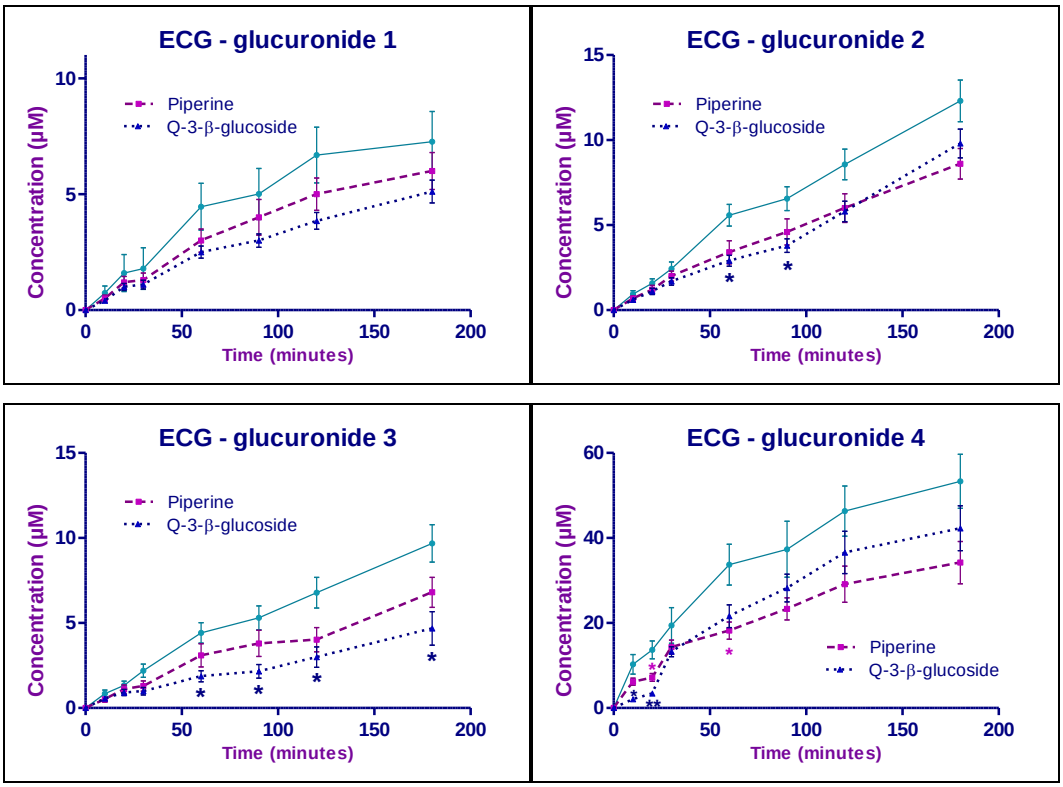

*Figure 6.6 – ECG glucuronidation with intestinal microsomes in the presence of Quercetin-3-β-glucoside and Piperine. Values are expressed as the mean ± SD (n = 2). *P < 0.05, ** < 0.01.*

6.4 – Discussion

In this study, the aim was to determine the inhibitory effects of two common dietary compounds, Quercetin and Piperine on glucuronidation using human microsomes. In previous studies, Piperine demonstrated inhibition of glucuronidation by inhibiting gastrointestinal transit of solids/liquids in rats and mice, inhibition of 3-hydroxybenzo(a) pyrene glucuronidation, and also significantly increased the bioavailability of curcumin (Bajad et al., 2001, Shoba et al., 1998, Singh et al., 1986). Lambert *et al* showed that EGCG glucuronidation was significantly inhibited by Piperine, and that it increased bioavailability in mice (Lambert et al., 2004). Quercetin had also previously shown to have inhibitory effects on both intestinal and liver microsomal glucuronidation (Mohamed and Frye, 2010) with IC_{50} values of 5.8 and 19.1 µM respectively. Based on these findings, it was hypothesised that EGCG, EGC, ECG, and EC glucuronidation would also be significantly inhibited by Piperine and Quercetin.

Glucuronidation by liver microsomes was not observed in the UGT-Glo assay with neither Quercetin nor Piperine. However, both compounds significantly inhibited glucuronidation via intestinal microsomes. This finding was probably due to differing UGT isoforms present in the two microsomal fractions, UGT1A8 and UGT1A10 being the intestine-specific isoforms (Tukey and Strassburg, 2000).

EGCG was co incubated with intestinal microsomes in the presence of 106.4 µM Piperine and showed a reduction in glucuronidation of between 27.2 % and 48.1 %. However, these values were not statistically significant as calculated by a one way ANOVA combined with Tukey's test, potentially due to the fact that the experiment was only carried out in duplicate. However, the results agree with the study carried out by Lambert et al, which showed a 40 % inhibition of glucuronidation of EGCG when co incubated with 100 µM Piperine (Lambert et al., 2004). Following this, the assay was carried out with Quercetin (15.2 µM) as it had also shown significant inhibition of intestinal glucuronidation the UGT-Glo assay. Co-incubation with Quercetin resulted in statistically significant inhibitory effects on EGCG glucuronide formation at 20 and 30 minutes of 42.1 % and 62.1 %

respectively. Although not statistically significant from one another, Quercetin offered a greater UGT inhibitory effect than Piperine on EGCG glucuronide formation. This was most likely due to direct competition, as Quercetin has a similar structure to catechins, and a glucuronide of Quercetin was also detected in the assay via LC/MS. It must be stressed that the Quercetin added to the enzyme assays was in its glucoside form (Quercetin-3-β-glucoside), which is generally how it appears in dietary sources, and this is not to be mistaken with Quercetin-glucuronide which was the UGT mediated conjugate detected after incubation with intestinal microsomes.

The results from these two compounds with EGCG suggest that inhibition of intestinal EGCG glucuronidation can potentially increase plasma concentrations of EGCG. Inhibition of glucuronidation can be more distinct in the case of Quercetin where there is competition for UGT enzymes, and enzyme co-factors such as UDP-glucuronic acid. Whereas Piperine mediated glucuronide inhibition of EGCG was compensated for by increased methylation of EGCG in HT-29 cells (Lambert et al., 2004). This wouldn't affect the bioavailability as there would still be very similar concentrations of metabolites formed and therefore in the plasma.

Similar results were observed with ECG. Quercetin appeared to have a greater inhibitory effect on glucuronidation on three glucuronides; however, the major glucuronide had a greater inhibition with Piperine over the 180 minutes of the assay. Over the first 20 minutes, Quercetin was visibly better at inhibiting glucuronide formation than Piperine. However Piperine became the major inhibitor at 60 minutes up until the end of the assay. This could be explained by the degradation of Quercetin over the assay period. However, Quercetin could have been extensively glucuronidated in the assay, as was seen with the formation of a Quercetin-glucuronide, which in turn had a lesser inhibitory effect on glucuronidation of the catechins.

EGC and EC in contrast to EGCG and ECG demonstrated that Piperine had a greater inhibitory effect on their respective glucuronide formation. Both had two glucuronides each in the UGT assay with intestinal microsomes but only one showed glucuronidation inhibition with Piperine and Quercetin.

This may be explained by the fact that they were present in very small concentrations compared to the major glucuronide product of EGC and EC. Interestingly, both EGC and EC showed statistically significant reductions in glucuronide conjugation. Piperine reduced glucuronidation of EGC by 72.1 – 78.9 %, and Quercetin 42.4 – 75.1 %. EC glucuronidation was inhibited by 66.4 – 78.2 %, and Quercetin inhibited glucuronide formation by 35.3 – 52.6 %. The difference in glucuronidation inhibition by Piperine and Quercetin towards the gallated and non-gallated catechins was unexpected. As the gallated catechins were used first in the experiments, it was assumed that the non-gallated catechins would also follow the inhibition properties demonstrated by Piperine and Quercetin; the non-gallated catechins are more extensively conjugated *in vivo*, hence would be more susceptible to inhibitory effects of other compounds. The use of intestinal microsomes in this study, and not intact cells also allowed us to determine that the inhibition was via direct actions on the UGT enzymes and not via uptake / efflux cellular transporters.

The results in our study demonstrate that common dietary compounds co-administered can decrease metabolism of green tea catechins significantly. This can potentially increase the bioavailability of the more bioactive parent catechins to circulate in the plasma and exert their beneficial health effects. The increase may be primarily due to inhibition of glucuronidation, and not necessarily due to increases in absorption. In the case of both Quercetin and Piperine it would be worthwhile to carry out detailed studies in cell systems also to see exactly which components are affected by the inhibitory compounds. And indeed the same should be done for other common food ingredients that are consumed regularly by the general population, so they can also be investigated for their potential health benefits, either via direct health effects, or by inhibiting the metabolism of beneficial polyphenols/ compounds in the diet.

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Chapter 7 – General discussion and future directions

7.1 – Summary of thesis findings

The aim of this thesis was to determine the absorption and potential health effects of green tea catechins in the oral cavity, metabolism of catechins in different enzyme fractions, protective effects of catechins and their metabolites against H_2O_2 induced mitochondrial DNA damage in intestinal cells, and inhibition of catechin metabolism by common food ingredients.

The key findings from chapter 3 were that green tea significantly reduced intracellular ROS levels in human oral cells, all four main catechins were significantly absorbed in human H400 oral cell lines, and all four catechins had significant protective effects against mtDNA damage in oral H400 cells.

The results from chapter 4 showed that green tea catechins were not significantly sulfated using liver s9 and cytosolic fractions, and that glucuronidation using intestinal and liver fractions showed the highest metabolism in our experimental systems. Methylation via porcine COMT significantly metabolised EGCG and EGC, and to a smaller extent EC. No methylated metabolites of ECG were detected. Methylation of EGCG at the 4' position (4'O-methyl-EGCG) greatly enhanced its glucuronidation via human liver microsomes. This was also the case for 4'-O-methyl-EGC where methylation at the 4' position greatly increased glucuronidation via liver microsomes, and sulfation via human liver cytosol. When EC was methylated at the 3' position, its capacity for sulfate conjugation greatly increased.

The key findings in chapter 5 showed that incubating physiological concentrations of catechins with intestinal cells for 4 hours conferred significant protection against H_2O_2 induced mtDNA damage. Metabolites of catechins showed reduced bioactivity but still significantly protected mtDNA against H_2O_2 induced damage.

In chapter 6 it was demonstrated that Piperine and Quercetin both significantly reduced the glucuronidation of EGC and EC. Glucuronidation of EGCG and ECG was also visibly reduced, however was not statistically significant for all individual metabolites. Piperine was a more effective inhibitor than Quercetin for the non-gallated catechins, and Quercetin was more effective in the case of the gallated catechins.

7.2 – Absorption and health effects in the oral cavity

In this study the effects of green tea catechins on protection against mitochondrial DNA damage were analysed, and to what extent catechins were absorbed into oral cells. Human H400 oral cell lines and buccal mucosal cells from human volunteers were used. The results showed extensive absorption of catechins in the oral cells after incubation with catechins for up to 60 minutes. EGCG and ECG were more extensively absorbed than EGC and EC, although the latter both were also absorbed in significant amounts. This was in agreement with previous studies which indicated high concentrations of catechins present in the saliva of subjects consuming 2-3 cups of green tea (Yang et al., 1999), and chewing green tea leaves (Lee et al., 2004). Both these studies however did not measure uptake of catechins in the oral cells, which was the novel approach in our experiments. Such uptake of catechins in oral cells led us to conclude that there is significant antioxidant protection against other potentially harmful compounds such as carcinogens from smoking or environmental pollution. The results from the mtDNA assay showed significant reductions in H_2O_2 induced damage in H400 cells, which indicated that the absorbed catechins are present in sufficient concentrations to prevent damage to mitochondria in the oral cavity. This agreed with previous studies which also showed positive effects of green tea catechins in the oral cavity (Zhang and Kashket, 1998, Yamamoto et al., 2003, Tsao et al., 2009, Maruyama et al., 2011, Magalhaes et al., 2009, Hirasawa et al., 2006, Ferrazzano et al., 2011), however these studies also did not measure intracellular levels of catechins in the oral mucosa. The concentrations used for the absorption studies and mtDNA

assays reflect the physiological concentration ranges that can be obtained from 2 – 3 cups of green tea. The oral mucosa is a good model system to study for its potential health benefits when exposed to catechins because it is one of the very few areas of the GI tract which is directly exposed to high concentrations of intact polyphenols in a physiological manner. It is worth noting that there were no conjugated metabolites observed in our experiments in oral cells. This was expected as there are no reported phase II enzymes expressed in the oral mucosa. The results from the study taken together indicate a potential role for beneficial health effects in the oral cavity; however the mechanisms of action at the molecular level still need to be studied further. Of course more in depth studies must be carried out in order to determine the exact *in vivo* situation occurring, which would help to ascertain exactly what concentrations of catechins are present inside the buccal cells and not just in the saliva. Another good avenue to explore would be to determine if catechins are actually adsorbed onto buccal cells in humans, as this would give a good starting point to the designing of strategies to have a constant stream of catechins being delivered to the oral cavity and GI tract, which could be in the form or sweets, beverages, or chewing gum.

7.3 – Protective effects of catechins and their metabolites against H_2O_2 induced mtDNA damage

Polyphenols are extensively metabolised via the action of phase II enzymes when ingested, and these metabolites have been shown to retain some bioactivity, albeit decreased when compared to their parent compounds. (Zhu et al., 2000, Lambert et al., 2007). The aim of this study was two-fold; to determine the activity of catechins and their metabolites, and protection against H_2O_2 induced mitochondrial DNA damage in intestinal cells. This is because mtDNA is more susceptible to ROS induced damage than nuclear DNA, and has less efficient repair mechanisms in place (Tulah and Birch-Machin, 2013, Alexeyev, 2009). Intestinal cells like oral cells are exposed to high concentrations of intact flavonoids after ingestion, however there is also extensive conjugation

which takes place in the small intestine (Walle et al., 1999, Vaidyanathan and Walle, 2002, Spencer, 2003, Hu et al., 2003). It was hypothesised, based on the studies in the oral cavity, that catechins would also significantly protect intestinal cells against mtDNA damage, and that if the metabolites showed any protective effects; they would be lower than the parent compounds. The results showed that physiological concentrations of catechins significantly protected mtDNA against damage in these cells. There was one instance when comparing the effectiveness of a parent catechin against its metabolite (EGCG vs 4'-methyl-EGCG), where the metabolite showed a slightly greater bioactivity, although this was not statistically significant. The rest of the catechins and their metabolites showed the expected outcomes of reduced bioactivity with conjugation (chapter 5). It is important to note here that although the intestine is exposed to high levels of flavonoids, their absorption is low (Walle et al., 1999, Walgren et al., 2000, Vaidyanathan and Walle, 2003, Vaidyanathan and Walle, 2001, Chan et al., 2007), which is why lower concentrations were used in this study. A major improvement in understanding the biological effects of catechins in intestinal cells is required. For this, future research would need to ascertain exact uptake concentrations of the targets flavonoid, and elucidate exact mechanisms by which they and their metabolites act to protect cells against mtDNA damage at physiological concentrations. Another avenue which will greatly improve our understanding of the bioactivities of metabolites would be the use of purified metabolites, which exact structures also known. This has important implications for the maintenance of good GI tract health, as there is potential for oxidative damage from environmental and ingested toxins in the intestinal epithelium. The studies can be further extended to other tissues which are constantly under oxidative stress, and can assess the protective effects, if any, against mtDNA damage. And as higher concentrations of catechins are required for antioxidant activities, it is unlikely that the protective effects are solely due to their direct ROS scavenging actions. What is more likely however, is the activation of some signalling pathways which allow the target tissues/cells to increase

the expression of innate cellular defences. This is of course needs to be studied in depth to elucidate exact mechanisms and signalling pathways affected.

7.4 – Inhibition of glucuronidation of catechins by Piperine and Quercetin

A previous study carried out by Lambert *et al* showed that bioavailability and metabolism of EGCG was favourably altered when co-administered with the purine alkaloid Piperine (Lambert et al., 2004). They did not however report on any differences in bioavailability and metabolism of the other three major catechins found in green tea. Our results were in agreement in regards to Piperine only inhibiting intestinal glucuronidation of EGCG and not liver glucuronidation. It was shown in this study for the first time that Piperine (106.4 µM) also reduced the metabolism of EGC and EC significantly over 90 and 180 minutes respectively. Although the results showed visibly distinct reductions in metabolism of ECG in the presence of Piperine, this was not deemed statistically significant.

Another known dietary inhibitor of glucuronidation was also tested for its ability to inhibit catechin metabolism in liver and intestinal microsomes. Consistent with the results obtained with Piperine, Quercetin also selectively inhibited intestinal glucuronidation and had no effect on liver microsome metabolism. Our results showed that Quercetin was also efficient at inhibiting the metabolism of catechins, albeit at a much lower concentration (15.2 µM) than Piperine. Quercetin was more effective than Piperine in reducing EGCG glucuronidation, possibly due to the fact that it directly competed with the catechin for the UGT active sites, being a flavonoid itself. This was confirmed with the detection of a Quercetin-glucuronide in all catechin incubations. In contrast, Piperine was shown to be a better inhibitor of EGC and EC glucuronidation than Quercetin. In the case of ECG, two of its metabolites showed less glucuronidation in the presence of Quercetin, one in the presence of Piperine, and one was reduced approximately the same. The results from this study indicate that less expensive and complicated strategies can be used to enhance

bioavailability of catechins. This is in contrast to strategies which aim to modify catechins before ingestion, encapsulation in various food matrices, and the use of nano-particle based delivery systems (Yao et al., 2015, Zhang et al., 2011). Further studies should also determine whether or not other common food ingredients can inhibit metabolism of target flavonoids, and if they have the same effects in vivo. This would greatly enhance our understanding of synergistic effects of polyphenols consumption in humans.

7.5 – Conclusions

The results from our studies provide a potential role of green tea catechins for maintaining good oral and intestinal health. They can act as preliminary studies in the preparation of more detailed and rigorous experiments which will help our understanding of polyphenol interactions with cells and other compounds, to determine precise mechanisms of action and cellular targets, and establish exact concentrations which can reach target tissues/cells. Future studies should use more physiologically representative catechins, i.e., conjugated catechins which are present in vivo and at precise concentrations that they are present in a particular target tissue, this will give a more comprehensive understanding of the potential effects of catechins in humans. Another relevant avenue of exploration is the precise effects on colon tissues as significant amounts of catechins pass through the intestine where they reach the colon and undergo metabolism via the colonic microflora, which are also important in determining exact biological effects of tea catechins.

Of course one of the limitations of our study was that purified metabolites were not used in some experiments, and future studies should take this into account, especially when trying to extrapolate *in vitro* results with *in vivo* health effects. This would greatly aid in translating the biological effects *in vitro* with a mechanistic understanding of target tissues *in vivo*.
Another limitation to overcome in future studies whether *in vitro* or *in*

vivo would be to design longer-term and better defined studies, which can incorporate the true effect of chronic, physiologically relevant exposure to catechins.

7.6 – References

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