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Formulation with ascorbic acid and sucrose modulates catechin bioavailability from green tea

Catrina M. Peters[1], **Rodney J. Green**[2], **Elsa M. Janle**[1], and **Mario G. Ferruzzi**[1,2,*]

[1]Department of Foods and Nutrition, Purdue University, West Lafayette, IN 47907

[2]Department of Food Science, Purdue University, West Lafayette, IN 47907

Abstract

In order to investigate the impact of common food ingredients on catechin absorption, green tea (GT) extract (50 mg) was formulated plain, with sucrose (GT+S), with ascorbic acid (GT+AA) and with sucrose and ascorbic acid (GT+S+AA). Bioavailability and bioaccessibility were assessed in Sprague Dawley rats and an *in vitro* digestion/Caco-2 cell model respectively. Absorption of epigallocatechin (EGC) and epigallocatechin gallate (EGCG) was significantly ($P<0.05$) enhanced in GT+S+AA formulations ($AUC_{0\text{-}6h}$= 3237.0 and 181.8 pmol*h/L plasma respectively) relative to GT control ($AUC_{0\text{-}6h}$ = 1304.1 and 61.0 pmol*h/L plasma respectively). *In vitro* digestive recovery was higher for EGC and epicatechin (EC) (~51-53%) relative to EGCG and epicatechin gallate (ECG) (< 20%) and was modestly enhanced in GT+S and GT+S+AA formulations. Accumulation of EGC, EGCG and ECG by Caco-2 cells was significantly ($P<0.05$) higher from GT+S+AA compared to other formulations while retention of catechins was enhanced in presence of ascorbic acid. These data suggest that formulation with sucrose and ascorbic acid may improve catechin bioavailability by enhancing bioaccessibility and intestinal uptake from tea.

Keywords

Bioavailability; Caco-2; Catechin; In vitro digestion; Green tea; Sprague Dawley

Introduction

Epidemiological studies have associated tea (*Camellia sinensis*) consumption with prevention of several chronic and degenerative diseases including cancer (Nichenametla, Taruscio, Barney, & Exon, 2006; Sun, Yuan, Koh & Yu, 2006), cardiovascular disorder (Kuriyama et al., 2006) obesity and diabetes (Wu, Lu, Chang, Chang, Wang & Chang, 2003; Iso, Date, Wakai, Fukui & Tamakoshi, 2006). Catechins (Figure 1), a class of polyphenols, are believed to be one of the physiologically active agents in tea due primarily to their abundance in brewed tea and tea beverages and reported biological activities in animals and humans including: antioxidant activity and modulation of plasma lipid profiles(Erba, Riso, Bordoni, Foti, Biagi & Testolin, 2005), enhancing vasodilatation (Kim et al., 2007), increasing fatty acid oxidation and insulin sensitivity (Venables, Hulston, Cox & Jeukendrup, 2008). With evidence

*Author to whom correspondence should be addressed: Dr. Mario G. Ferruzzi, 745 Agriculture Mall Dr., West Lafayette, IN 47907, Phone: (765) 494-0625, Fax: (765) 494-7953, mferruzz@purdue.edu.

suggesting a potential role for catechins in prevention of chronic disease, interest in the bioavailability of these polyphenols from tea and tea based foods and beverages has intensified.

Bioavailability of catechins from tea is believed to be relatively poor. In humans, maximum catechin plasma concentrations of up to 1-2 mol/L are achieved between one and two hours after consumption followed by rapid clearance restoring plasma concentrations to baseline levels within 24 hours of initial consumption (Leenen, Roodenburg, Tijburg & Wiseman, 2000; Chow et al., 2005; Henning, Choo & Heber, 2008). Several factors are believed to limit bioavailability of intact catechins including: 1) potential catechin sensitivity to digestive conditions (Record & Lane, 2001; Green, Murphy, Schulz, Watkins, & Ferruzzi, 2007b); 2) poor intestinal transport (Vaidyanathan & Walle, 2001; Vaidyanathan & Walle, 2003; Zhang, Zheng, Chow & Zuo, 2004); and 3) rapid metabolism and clearance (Chow et al., 2005). Previously, exposure of green tea to simulated digestive conditions was found to reduce total catechin levels by 80% and 90% relative to undigested controls (Record & Lane, 2001; Green et al., 2007b), with the majority of catechin degradation believed to occur under small intestinal conditions where elevated pH and presence of reactive oxygen species (Parks, 1989) provide favorable conditions for catechin auto-oxidative reactions (Tanaka & Kouno, 2003; Neilson, Hopf, Cooper, Pereira, Bomser & Ferruzzi, 2007). Following the potential for significant digestive loss, transport of catechins in the intestine is limited by their affinity to efflux transport systems including Multidrug Resistance Proteins (MRP) and P-glycoprotein (PgP) known to limit uptake of many xenobiotics including catechins (Jodoin, Demeule & Beliveau, 2002; Vaidyanathan & Walle, 2003; Zhang et al., 2004). Combined, poor intestinal stability and intestinal transport may serve to limit absorption of intact catechins following oral consumption of tea.

More, recently, formulation of green tea with ascorbic acid and ingredients containing sugars and ascorbic acid such as citrus juices were found to significantly enhance catechin stability to simulated digestive conditions (Green et al., 2007b)and alter accumulation of catechins by human intestinal cells (Caco-2) in culture (Green, Murphy & Ferruzzi, 2007a). While promising, the extent to which beverage formulation may influence catechin bioavailability *in vivo* remains unclear. In order to expand upon previous *in vitro* work, the objectives of the current study were to assess the impact of ascorbic acid and sucrose on catechin bioavailability *in vivo* and to determine the extent to which an *in vitro* digestion/Caco-2 human intestinal cell culture model may predict catechin bioavailability *in vivo*.

Materials and Methods

Chemicals and standards

Green tea water soluble extract was obtained as a gift from Nestlé Product Technology Center (Marysville, OH). All beverages were formulated with 50 mg green tea water extract providing 20.4, 5.1, 26.5, and 4.0 μmoles of epigallocatechin (EGC), epicatechin (EC), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) respectively. L-Ascorbic acid was obtained from VWR (West Chester, PA). Sucrose (>99% pure) was obtained from Research Products International Corp (Mt Prospect, IL). β–glucouronidase with sulfatase enzymes (G0751-1MU) and EC, EGCG, EGC and ECG standards were obtained from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC) grade solvents were obtained from J.T. Baker (Phillipsburg, NJ). Isoflurane was obtained from Baxter Healthcare Center (Deerfield, IL).

Green Tea Formulations

Specific tea formulations used in this study were selected based on previous *in vitro* experiments illustrating a positive impact of ascorbic acid and citrus juices on both digestive

stability and cellular accumulation and retention (Green et al., 2007a; Green et al., 2007b). Overall, four test formulations were prepared for *in vitro* digestion, cell culture and *in vivo* bioavailability experiments in the same manner and with the same quantity of green tea extract. Green tea only (GT), green tea+sucrose (GT+S), green tea+ascorbic acid (GT+AA), and green tea+sucrose+ascorbic acid (GT+S+AA) samples were formulated as described in Table 1. All treatment groups contained identical amounts of green tea extract (50 mg) providing identical catechin profiles. Additionally, the ratios of green tea solids to sweeteners and ascorbic acid selected are typical of commercial ready-to-drink (RTD) beverages. Formulations were solubilized in 15 mL of double distilled water and 1.5 mL of physiological saline (0.9% NaCl) for *in vitro* digestion and *in vivo* gavage administration respectively. Solutions were prepared fresh and utilized immediately upon preparation. Aliquots were flushed with nitrogen gas and stored frozen at -80°C for subsequent analysis.

Catechin *in vivo* bioavailability

All animal protocols were reviewed and approved by the Purdue Animal Care and Use Committee. Catechin bioavailability was assessed using male Sprague Dawley rats (n=8 per treatment group; ~275g weight) obtained from Harlan (Indianapolis IN). Rats were given Harlan 2014 diet and deionized water ad lib upon arrival and allowed to acclimatize for three days. Following the acclimation period rats were placed under anesthesia with isoflurane and a polyethylene catheter was implanted into the jugular vein. Burpernex analgesic support was administered prior to animals regaining consciousness. Patency of catheters was maintained by flushing every 12 hr with Heplock® solution. Animals were allowed 24 hr of recovery post surgery.

Food was restricted 6 hr prior to gavage and was offered 2 hr after administration of tea formulations. Green tea formulations were solubilized in 1.5 mL of 0.9% saline and administered by intragastric gavage with an 18 gauge stainless steel gavage needle and plastic syringe. Four hundred μL of blood was collected from the jugular catheter into heparinized tubes at 0, 30, 60, 120, 240 and 360 min post-gavage, centrifuged at 4,000 rpm for 10 minutes at 4° C. Two hundred μL of resulting plasma was collected and combined with 50 μL acidified saline (2% ascorbic acid wt/wt), purged with N_2 and stored at -80° C until analysis.

In Vitro Digestion

Catechin digestive stability was assessed using a two-stage *in vitro* digestion model originally described by (Garrett, Failla & Sarama, 1999) with modification to gastric and small intestinal conditions in order to better replicate previously reported rodent GI conditions including approximate gastric/small intestinal volumes and pH (Kararli, 1995; McConnell, Basit & Murdan, 2008). Briefly, GT formulations (Table 1) were dissolved in 1.5 mL of saline and brought to a total volume of 9 mL with 0.9% saline and the initial pH was recorded. The gastric phase of digestion was initiated by addition of 0.9 mL porcine pepsin solution (40 mg/mL in 0.1 N HCl) and adjustment of the pH to 2.5±0.1 with 1.0 N HCl. Samples were blanketed with N_2 and incubated at 37° C in a covered shaking water bath for 1 hr. The small intestinal phase was initiated by addition of 100 mM $NaHCO_3$ followed by addition of small intestinal enzyme solution [1.35 mL of porcine lipase (1.0 mg/mL) and pancreatin (2.0 mg/mL) in 100 mM $NaHCO_3$, and 1.35 mL bile (12.0 mg/mL) in 100 mM $NaHCO_3$]. The final sample pH was adjusted to 5.5±0.1 with 1.0 N NaOH, and final reaction volume standardized to 15 mL with 0.9% saline. Samples were then purged with N_2 and placed in a 37° C shaking water bath for 2 hr. After incubation, aliquots of digesta were centrifuged at 10,000 × g (4° C) for 1 hr to separate the aqueous bioaccessible fraction from insoluble residues. Aliquots of starting material, crude digesta and filtered (0.2 m) aqueous fraction were collected and stored -80° C until analysis.

Caco-2 Intestinal Cell Culture

Catechin intestinal accumulation and retention studies were conducted using the clonal line of Caco-2 (TC7) between passages 81-93. Cells were grown and differentiated in 6-well plastic dishes in a humidified atmosphere of air/CO2 (95:5) at 37°C. Catechin uptake experiments were performed at 14 days post confluency. Briefly, catechin test media was prepared by combining filtered aqueous fraction from *in vitro* digestions with phosphate buffered saline (PBS) (1:13) followed by adjustment to pH 5.0. Monolayers were washed once with PBS before adding test media. Cultures were then incubated at 37° C for 3 hr. Following incubation, culture medium was removed by aspiration and monolayers were washed twice with ice-cold PBS. Replicate cultures were washed and fresh complete catechin free Dullbeco's modified eagle media (DMEM) was added before being returned to the incubator for 1 hr at 37° C to determine retention of catechins by Caco-2 cells. Following incubation cells were washed twice with cold PBS and collected by scraping into 1.0 mL of ice-cold PBS at pH 5.0 with 0.1% ascorbic acid, flushed with nitrogen gas and stored at -80° C until analysis.

Catechin Extraction

Catechins were extracted from sonicated Caco-2 cells with 3 mL ethyl acetate (0.01% BHT). Extraction was repeated a total of three times and ethyl acetate layers were pooled, dried under vacuum and resolubilized in 300 μL mobile phase. Catechin extraction from 100 L of rat plasma was initiated by addition of 1 mL of enzyme solution (250 U β-glucuronidase with sulfatase activity in 0.4 M NaH_2PO_4 pH 4.5) to degonjugate glucuronide and sulfate metabolites. Samples were purged with N_2 incubated in 37° C shaking water bath for 45 min and then extracted with 3 mL ethyl acetate (0.01% BHT) a total of three times. Organic extracts were pooled, dried under vacuum and resolubilized in 200 μL mobile phase prior to analysis by HPLC.

HPLC analysis

Catechins from digesta, cells and plasma were quantified by HPLC with electrochemical detection (ECD) as described by (Neilson et al., 2007) with minor modification. Briefly, a HP1050 LC system (Agilent Technologies; Santa Clara, CA) equipped with an 8-channel ESA CoulArray electrochemical array detector (ECD) (Chelmsford, MA) was used. Catechins were resolved on a Waters Xterra C18 (3.9mm i.d. × 100mm) reversed phase column (Milford, MA) by binary gradient elution with the following mobile phases: phase A (ddH_2O, ACN, and formic acid (91.9/8/0.1 v/v)) and phase B (ddH_2O, ACN, methanol (MeOH) and formic acid (69.9/27/3/0.1 v/v). System flow rate was 0.8 mL/min and the column was thermostated at 25° C. Peak identification was confirmed by co-chromatography with authentic standards of EC, EGC, EGCG and ECG. Quantification was conducted using multi-level response curves constructed using the cumulative response at 60mV, 130mV and 200mV.

Data analysis

All statistical analyses were completed using SAS for Windows version 9.1.3. Catechin content in starting formulations and final digesta were expressed as mean ± standard error of mean (SEM) of five independent observations. Caco-2 intracellular catechin content was expressed as mean ± SEM of four independent observations. Plasma pharmacokinetic response curves were constructed from plasma catechin levels calculated from a final sample size of n=7 (GT), n=7 (GT+SUC), n=7 (GT+AA), and n=6 (GT+SUC+AA). The maximum catechin plasma concentration (C_{max}) and time of maximum catechin plasma concentration (T_{max}) were obtained directly from the individual plots of plasma catechin concentration versus time. Plasma area under the curve values from 0 to 6 hr ($AUC_{0\text{-}6h}$) were calculated using the linear trapezoidal rule. Group differences were determined by analysis of variance using Fisher's PLSD post-hoc test (< 0.05).

Results and Discussion

Catechin Plasma Pharmacokinetics from formulated GT

To determine how GT formulations would affect *in vivo* catechin bioavailability, four GT formulations were administered to male Sprague Dawley rats by oral gavage and catechin plasma pharmacokinetics determined over 6 hr. Individual catechin plasma pharmacokinetic profiles and parameters can be seen in Figure 2 and Table 2 respectively. Plasma catechin levels were found to increase sharply following oral gavage reaching maximum levels between 1 and 2 hr and returning close to baseline levels by 6 hr. Plasma levels of non-gallated catechins EGC and EC were significantly ($P < 0.05$) higher than gallated EGCG and ECG consistent with previous reports on the *in vivo* absorption of catechins from GT in rodent and human models (Chow et al., 2003;Chow et al., 2005;Henning et al., 2008).

Formulation of GT was found to significantly impact overall catechin bioavailability, measured as area under the curve ($AUC_{0\text{-}6h}$) (Table 2). Of the individual catechins, EGC was most affected by GT formulation. $AUC_{0\text{-}6h}$ for EGC increased from 1304.1±405.3 pmol/L*h for GT to 2569.3±518.2, 2775.1±438.1 and 3237.0±466.1 pmol/L*h for GT+S, GT+AA and GT+S +AA respectively. EGCG $AUC_{0\text{-}6hr}$ was significantly ($P<0.05$) higher in GT+S (155.0±58.2 pmol/L*h) compared to GT only (61.0±26.6 pmol/L*h) formulations and was further increased in GT+S+AA (181.8±17.1 pmol/L*h) formulations. EC $AUC_{0\text{-}6hr}$ was significantly ($P<0.05$) higher for non-sucrose containing formulations (GT and GT+AA) compared to GT+S or GT +S+AA.

Inclusion of sucrose to GT formulations (GT+S and GT+S+AA) had a distinct effect on catechin absorption profiles compared to sucrose free formulations (GT and GT+AA). Catechin absorption in rats administered GT only formulations appeared to be more rapid than from formulated treatments (Figure 2). Addition of sucrose (GT+S and GT+S+AA) resulted in a slight delay in T_{max} from ~1 to closer to 2 hr. This increase may have been due in part to the increase in viscosity of gavage solution due to the addition of sucrose. Sucrose formulations (GT+S and GT+S+AA) were observed to be have viscosities approximately six times higher than non sucrose containing formulations once solubilized in saline for gavage (data not shown). Increased viscosity is known to impact gastric emptying (Marciani et al., 2000) and by extension may influence kinetics of absorption for compounds in the small intestine. As stated before, the high sucrose level relative to green tea powder utilized in this study was chosen to emulate a typical commercial sweetened RTD green tea beverage. As beverages may be formulated with other non-caloric and caloric sweeteners using lower or higher usage rates, a more detailed study of the dose-dependent enhancement of catechin absorption by various sweeteners is required to determine if optimum ratios exist.

Formulation of GT with ascorbic acid (GT+AA and GT+S+AA) also altered plasma pharmacokinetic parameters. Specifically, an increase in $AUC_{0\text{-}6h}$ of EGC and EGCG relative to GT and GT+S formulations was observed for ascorbic acid containing formulations (Table 2). EGC and EGCG AUC were highest from GT+S+AA compared to other formulations. C_{max} of EGC and EGCG was also increased with sucrose and ascorbic acid but this effect only reached statistical significance for the GT+S+AA compared to the GT only formulation. T_{max} was not significantly impacted by formulation with ascorbic acid.

Several factors may have contributed to observed enhancement of *in vivo* bioavailability for specific catechins in formulated products relative to the GT only formulation. While sucrose increased viscosity of GT solutions, likely impacting GI transit time, it may also potentially enhance catechin uptake by the intestine. Schramm et al. (2003) previously demonstrated the ability of carbohydrate in the form of white bread and sucrose to enhance epicatechin bioavailability in humans. The mechanism associated with this increase in catechin

bioavailability has not been systematically studied. However, reports have indicated that GT catechins may bind to glucose transporters GLUT2 and SGLUT1 but do not appear to be transported by these proteins (Kobayashi et al., 2000; Johnston, Sharp, Clifford & Morgan, 2005) thereby potentially limiting their bioavailability. Enhancement of EGC and EGCG bioavailability by addition of ascorbic acid in combination with sucrose (Figure 2, Table 2) may involve stabilization of these labile catechins from oxidative degradation in the GI tract and/or a decrease in intestinal efflux of absorbed catechins back to the lumen (discussed further in the next sections). The ability of ascorbic acid to stabilize labile catechins in simulated digestive conditions (Green et al., 2007b) as well as collected tissues and fluids has been shown previously (Chen, Zhu, Wong, Zhang & Chung, 1998; Chu, Wang, Chu, Rogers, Choy & Pang, 2004). Additionally, ascorbic acid has previously demonstrated the ability to modulate PgP expression and activity in microbial systems, limiting efflux of xenobiotics from bacterial models (El-Masry & bou-Donia, 2003). Ascorbic acid was utilized in select formulations (GT +AA and GT+S+AA) formulations and in plasma collection for this study. While all plasma samples were treated identically, the additional ascorbic acid introduced by formulation may have increased plasma levels and by extension further stabilized collected plasma and therefore cannot be fully excluded as an additional factor. Combined, these data support the notion that formulation factors may have a significant impact on catechin absorption from tea based food or beverage products.

Impact of GT formulation on catechin *in vitro* digestive recovery

Based on *in vivo* results, *in vitro* digestion experiments were designed to better understand the potential mechanisms by which sucrose and ascorbic acid may have enhanced catechin bioavailability through modulation of digestive recovery (bioaccessibility). Simulated digestive conditions were modified from previous experiments with tea (Green et al., 2007b) to better mimic rodent digestive conditions thereby facilitating comparison to rodent *in vivo* data. In addition to moderate differences in gastric and small intestinal pH conditions, these adaptations resulted in a more concentrated digestive matrix (3.7 moles of total catechins per mL compared to ~2.0 moles total catechins per mL for a standard digestive reaction).

Simulated digestion of plain GT resulted in significant ($p<0.05$) loss of all catechins compared to starting undigested levels (Figure 3). Relative digestive recovery for individual catechins was EGC ≈ EC > EGCG > ECG. Gallated catechins EGCG and ECG were most affected by simulated digestion with a losses of 79.3% (26.5±0.7 to 5.1±0.3 moles/reaction) and 88.8% (4.0±0.1 to 0.4±0.04 moles/reaction), respectively. Non-gallated catechins EGC and EC were observed to be less sensitive with digestive losses of 43.6% (20.4± 0.5 to 10.9±0.7 moles/reaction) and 46.4% (5.1± 0.1 to 2.6±0.03 moles/reaction), respectively. These results indicate that the bioaccessibility of gallated catechins (EGCG and ECG) is significantly lower than EGC and EC in agreement with *in vivo* bioavailability observed in rodents (Figure 2, Table 2).

While in agreement with *in vivo* data these results differed from those previously reported for simulated gastric and small intestinal digestive recovery of green tea catechins using conditions approximating human gastro-intestinal conditions (Record & Lane, 2001; Neilson et al., 2007; Green et al., 2007b). These previous studies determined that EGC and EGCG were the most sensitive catechins while EC was found to be relatively stable to digestion. Difference in relative catechin stability observed in the current study and likely to lower intestinal pH (5.5 versus ~7.0-7.4) of this model relative to previous experiments (Green et al., 2007b). Parallel digestions of the identical formulation using conditions described by Green et al. (2007b) resulted in significant degradation of EGC and EGCG (data not shown), in agreement with these previous reports (Record & Lane, 2001; Green et al., 2007b). These data suggest that catechin digestive behavior might be altered by small differences in GI conditions such as those existing between rodent and human intestines.

Formulation of GT with sucrose (GT+S) and AA (GT+AA) modestly but significantly ($p<0.05$) improved overall catechin digestive recovery (Figure 3), increasing total catechin content in digesta from 19.1.0±0.8 μmoles/reaction to 23.2±1.0 and 20.0±0.4 μmoles/reaction respectively. Among individual catechin species, sucrose addition enhanced recovery of EGC from 10.9±0.7 to 12.2±0.5 μmoles/reaction for GT to GT+S digesta respectively. Addition of ascorbic acid increased EGC recovery to 11.9±0.1 μmoles/reaction and the combination of sucrose and ascorbic acid (GT+S+AA) further enhanced digestive recovery of EGC to 13.3 ±0.1 μmoles/reaction in final digesta. EC digestive recovery was not significantly improved by formulation with sucrose, AA or in combination. EGCG and ECG digestive recovery was improved by 41.5% and 46.3% respectively in GT+S compared to plain GT formulation (Figure 3). ECG had the lowest digestive recovery and was the least affected by formulation. ECG digestive recovery was determined to be 11.2% and 14.6% for GT and GT+S, respectively, compared to that of the starting material.

These results suggest only a modest effect of formulation to measures of catechin bioaccessibility in this modified *in vitro* digestion model with sucrose having the most impact. The positive impact of sucrose may be related, in part, to its preabsorptive effect on catechin solubility in rodent GI and in the present intestinal model. Concentrated polyphenol rich tea solutions are known to be susceptible to formation of precipitates composed of tea catechins, other polyphenols, caffeine (present in the green tea extract at 4.5 mg/50 mg extract), and other tea constituents including divalent cations such as calcium often found in suspension (Liang & Xu, 2003; Jobstl, Fairclough, Davies & Williamson, 2005). This phenomenon is often referred to as tea cream (in black tea) or flocculation (in green tea). Of the individual catechins present in green tea, less soluble gallated catechin (EGCG and ECG) have a stronger tendency to form precipitates compared to more soluble non gallated catechins (EGC and EC) (Liang, Lu & Zhang, 2002). Furthermore, these associations and subsequent precipitation of catechins and caffeine are known to form more readily under acidic conditions (Liang & Xu, 2001), such as those encountered in the gastric phase of digestion. The lower bioaccessibility of gallated catechins may be driven by similar interactions and may contribute, in part, the poor bioavailability of gallated catechins relative to non-gallated counterparts observed in the *in vivo* experiments (Figure 2, Table 2) and in previous rodent and humans studies (Chow et al., 2003; Chow et al., 2005; Henning et al., 2008). The presence of sucrose may serve to interfere with these associations and enhance catechin solubility in the GI thereby enhancing bioaccessibility of individual catechins. This subtle increase in bioaccessibility may have contributed partially to the modest increase in catechin bioavailability from cocoa when consumed with carbohydrate previously reported (Schramm et al., 2003) and the enhanced *in vivo* response observed for EGCG in this study (Figure 2; Table 2). Additional experiments are required to determine if an optimal level of carbohydrate based sweetener exist to optimize catechin intestinal solubility.

Impact of GT formulation on accumulation and retention of catechins by Caco-2 human intestinal cells

To better understand how *in vivo* absorption of catechins may have been influenced by formulation, catechin intestinal uptake was investigated by incubating (3h) differentiated Caco-2 monolayers. Test media was produced from filtered aqueous digesta for each tea formulation and diluted with PBS (pH 5.0). Formulations with sucrose and ascorbic acid alone did not significantly impact accumulation of individual catechins by Caco-2 human intestinal cells (Figure 4). However, EGC accumulation from GT+S+AA media was significantly ($P<0.05$) higher than GT+S at 144.0±21.2 pmol/mg and 54.7±7.8 pmol/mg respectively. Similarly, EGCG accumulation increased from 25.8±5.3 and 22.8±2.8 pmol/mg protein for GT and GT+S respectively to 93.2±9.9 pmol/mg for GT+S+AA, a 4-fold increase from GT

only. ECG also showed a similar trend to increase with GT+S+AA formulation, 1.9±0.5 to 5.6 ±1.7 pmol/mg for GT and GT+S+AA respectively.

Retention (1h) of catechins by Caco-2 cells was also assessed in order to determine if formulation would impact the efflux of these tea polyphenols by human intestinal cells. Intracellular retention of non-gallated catechins from GT only formulation was poor with EGC and EC intracellular levels observed to be 5.5±3.4 and1.3±0.8 pmol/mg respectively following incubation with fresh DMEM. These levels represent ~6% of the original amounts of EGC and EC accumulated over 3 h indicating a significant degree of efflux or metabolism of these catechins by Caco-2. In contrast, retention of gallated catechins was higher from plain GT formulation with levels of 8.3±18 and 0.4±0.1 pmol/mg, representing 32.4% and 24.2% of the original intracellular levels of EGCG and ECG respectively. While intracellular metabolism was not assessed in these studies ABC transporters such as PgP and MRP2 are known to transport catechins back to the apical compartment and limit retention and trans epithelial transport in three-compartment Caco-2 models (Vaidyanathan & Walle, 2003; Zhang et al., 2004). The affinity for catechins to these ABC transporters is believed to significantly limit absorption of these compounds from tea beverages and combined with the potential for intracellular metabolism likely explains the poor retention of catechins by Caco-2 in the present studies.

GT formulation with ascorbic acid was observed to directly influence retention of catechins by Caco-2 monolayers. GT formulations containing ascorbic acid (GT+AA and GT+S+AA) had significantly ($P<0.05$) higher catechin retention than GT or GT+S (Figure 4). EGC retention was significantly increased in GT+S+AA and GT+AA groups, 66.7±7.3 and 44.1 ±10.1 pmol/mg respectively compared to GT and GT+S at 5.5±3.4 and 7.2±2.8 pmol/mg respectively. EC retention was similarly enhanced in both GT+S+AA and GT+AA, groups with intracellular levels of 12.4 ± 2.0 and 8.2 ± 3.5 pmol/mg representing an increase in retention from 6.2% (GT) to 46.7% (GT+AA) and 52.9% (GT+S+AA) of the intracellular levels observed after 3h accumulation. Similarly, EGCG retention was highest in GT+S+AA formulations (44.6±3.8 pmol/mg) compared to GT+AA (20.0±2.1 pmol/mg), GT+S (13.2±1.8 pmol/mg) and GT alone (8.3±1.8 pmol/mg). EGCG retention was significantly ($P<0.05$) increased in ascorbic acid containing formulations by 140% (GT+AA) and 437% (GT+S+AA) compared to GT formulations respectively. The enhanced intracellular accumulation and subsequent retention of EGC and EGCG from GT +S+AA formulations is in general agreement with *in vivo* plasma pharmacokinetic data (Figure 2) suggesting that enhanced bioavailability of these catechins from GT+S+AA formulations is likely due, in part, to alteration of intestinal transport by ascorbic acid as well as enhanced bioaccessibility mediated by sucrose.

In contrast to the modest effects on *in vitro* digestive recovery in the present model, presence of ascorbic acid in GT formulations significantly altered catechin profiles of intestinal cells in culture. Previously, ascorbic acid has demonstrated the ability to modulate PgP expression and activity in microbial systems, limiting efflux of xenobiotics from bacterial models (El-Masry & bou-Donia, 2003). Human cell culture experiments have also shown addition of ascorbic acid limited the susceptibility of select cancer therapeutic agents to multidrug resistance effects by enhancing intracellular levels of these compounds (Chiang, Song, Yang & Chao,1994; Cai, Lu, Miao, Lin & Ding, 2007). Similar inhibition of PgP expression and/or activity by ascorbic acid in Caco-2 may contribute to the observed increases in catechin intracellular retention and this effect may explain the increase in plasma response observed in rodents (Figure 2). Potential synergies between ascorbic acid and catechins therefore merit further investigation as a strategy to enhance delivery and activity of catechins from foods and dietary supplements in humans.

While the coupled *in vitro* digestion/Caco-2 model appears promising as a screening tool for catechin bioaccessibility, it is important to note that when comparing intestinal uptake of

catechin in rodent models to accumulation and retention from human cell cultures, as done in these studies, some inter-species differences in intestinal transport and metabolism may exist. These differences may explain, in part, the discrepancies in EC and EGCG absorption observed between *in vitro*/cell culture and *in vivo* models (Figures 2 and 4; Table 2). Therefore, while useful as a method to predict the directional influence formulation may have on catechin absorption from tea beverages, quantitative comparison of the influence of formulation on pharmacokinetic parameters require more detailed investigation in animal models or humans.

Conclusions

The objectives of the current study were to assess the impact of GT formulation with ascorbic acid and sucrose on catechin bioavailability *in vivo* and to determine the extent to which a coupled *in vitro* digestion/Caco-2 human intestinal cell culture model can predict bioavailability *in vivo. In vivo* catechin bioavailability was impacted by formulation with absorption of EGC and EGCG greatest from formulations including both sucrose and ascorbic acid while EC bioavailability was greatest from plain green tea formulations. In all formulations, bioavailability of gallated catechins (EGCG and ECG) were lower than non-gallated (EGC and EC). *In vitro* digestive studies highlight solubility and stability as modulators of catechin bioaccessibility and may critical predictors of *in vivo* bioavailability. Caco-2 intestinal cell accumulation and retention of catechins suggested that enhancement of catechin bioavailability may be partially explained through modulation of intestinal uptake and transport. Combined these results suggest that the common practice of co-formulating ready-to-drink GT beverages with sucrose and ascorbic acid may enhance bioavailability of catechins through both pre-absorptive and absorptive mechanisms. Also, while quantitative *in vitro* bioaccessibility and Caco-2 data cannot be directly translated to *in vivo* situations, qualitative and directional changes induced by formulation appear to be predictable using these models. When used in conjunction with *in vivo* bioavailability assessments, these models may provide additional insights into the mechanism by which pre-absorptive events and/or intestinal absorption is altered *in vivo*.

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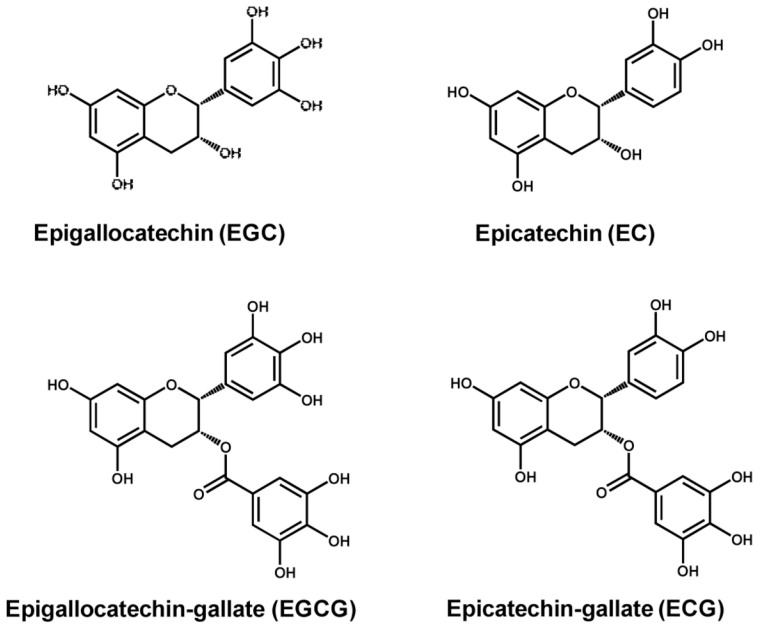

Figure 1.
Structures of the four major catechins present in green tea.

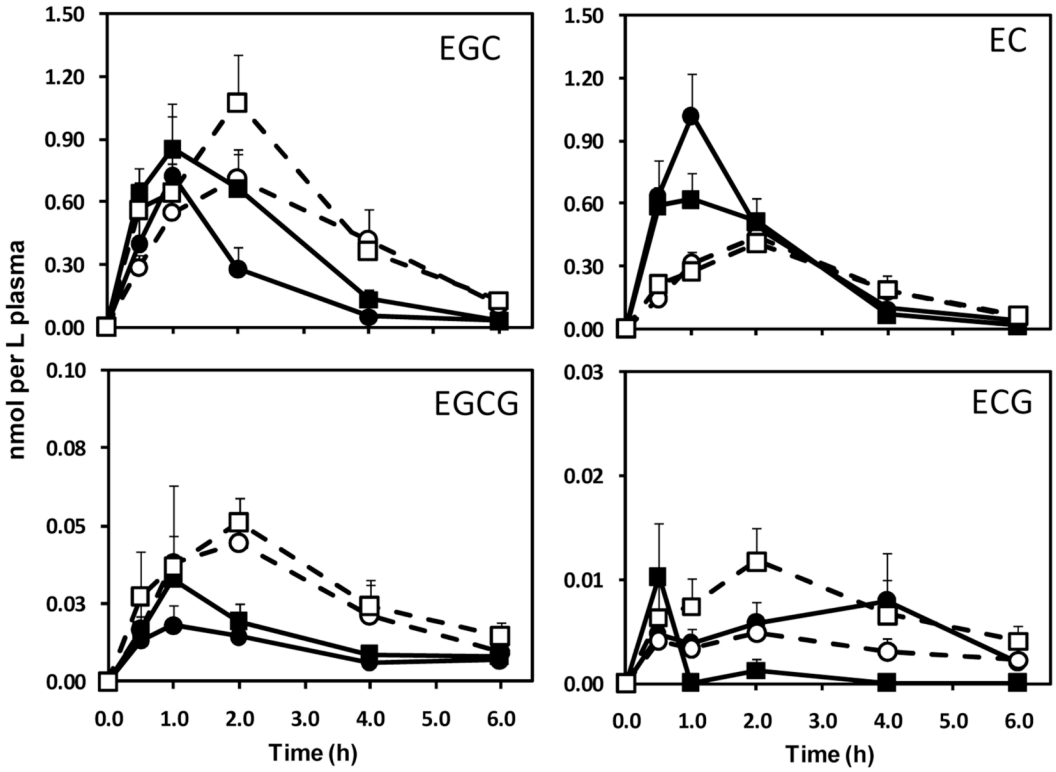

Figure 2.
Six hour plasma pharmcokinetic response of green tea catechins in Sprague Dawley rats following oral gavage of GT (●); GT+S (●); GT+AA (■); or GT+S+AA (□). Pharmacokinetic parameters including T_{max}, C_{max} and AUC can be seen in Table 2.

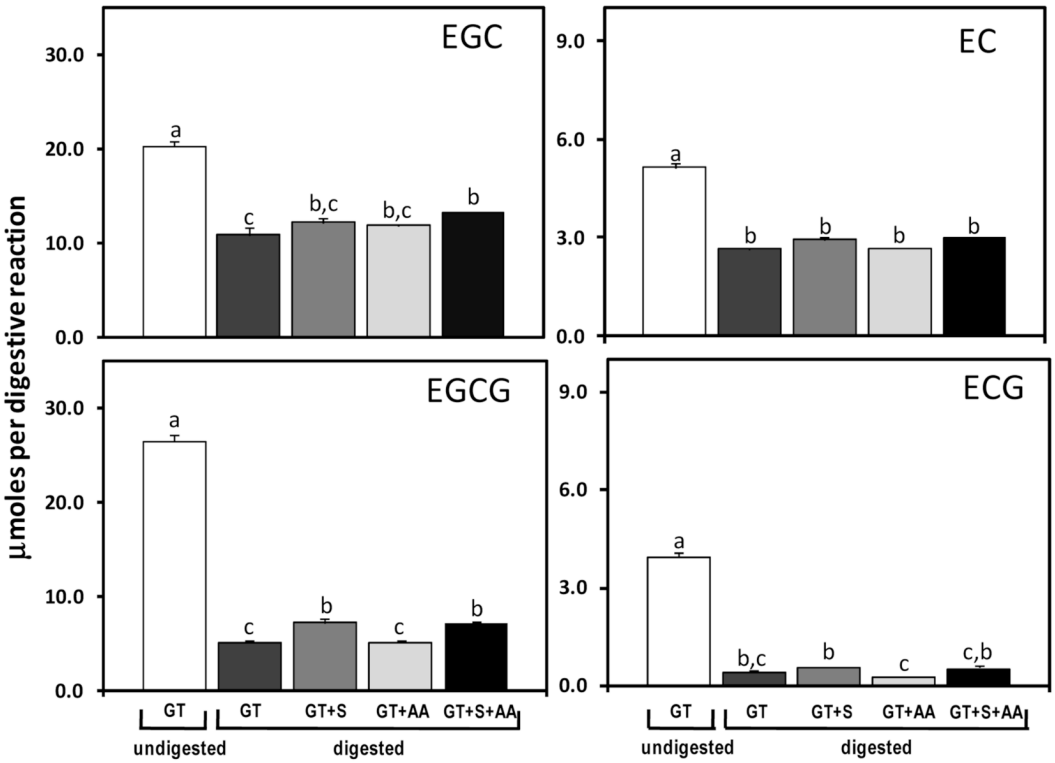

Figure 3.

Catechin profiles of GT test formulation before and after exposure to *in vitro* digestive conditions as described in *Materials and Methods*. Bars represent: pre-digested catechin content (□); and content in digesta of GT (■); GT+S (■); GT+AA (□); and GT+S+AA (■) formulations. Data expressed as moles of catechins per digestive reaction ±SEM (n=5). Presence of different letters indicate significant differences between treatments as determined by Fisher's LSD test ($P<0.05$).

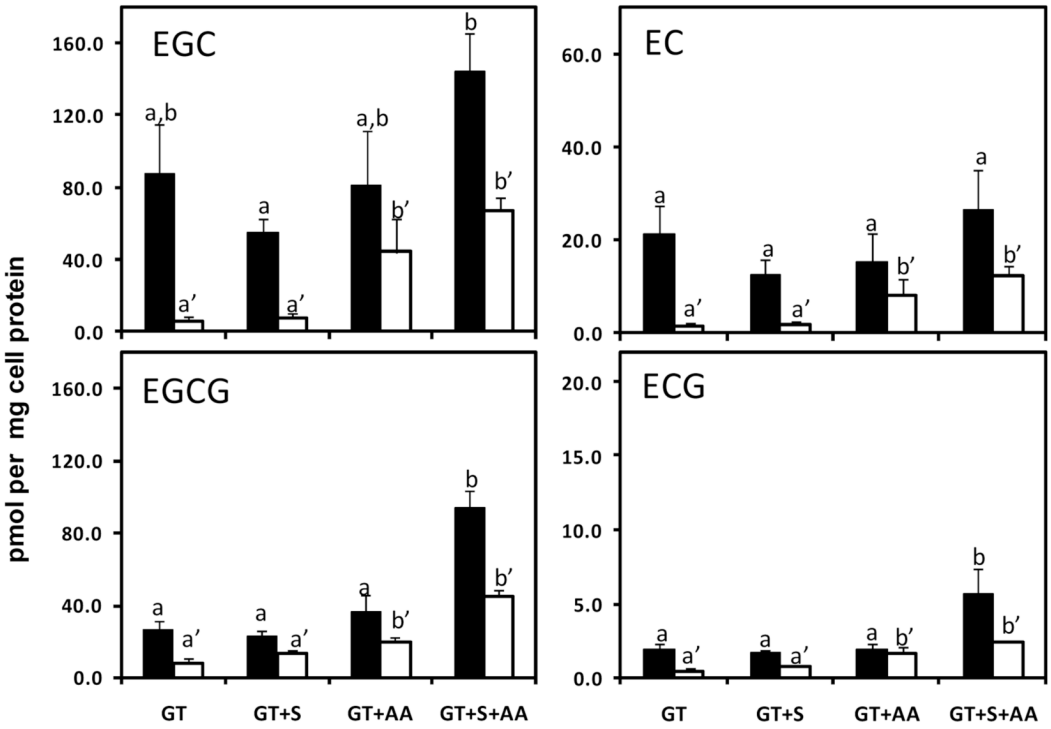

Figure 4.

Three hr accumulation (■) and subsequent 1 hr retention (□) of green tea catechins by differentiated monolayers of Caco-2 human intestinal cells. Data expressed as pmol catechin per mg cell protein ±SEM (n=4). Presence of different letters indicate a significant difference in catechin intracellular levels following either accumulation or retention between GT formulations as determined by Fisher's LSD test ($P<0.05$).

Table 1

Green Tea formulations utilized for parallel *in vitro* and *in vivo* experiments[1]

Ingredients (mg)	**GT**	**GT+SUC**	**GT+AA**	**GT+SUC+AA**
Sucrose (mg)	-	1,250.0	-	1,240.0
Ascorbic Acid (mg)	-	-	10.0	10.0
Green Tea Extract (mg)	50.0	50.0	50.0	50.0
Catechins (μmoles)				
EGC	20.4	20.4	20.4	20.4
EC	5.1	5.1	5.1	5.1
EGCG	26.5	26.5	26.5	26.5
ECG	3.9	3.9	3.9	3.9
Total Catechin	55.9	55.9	55.9	55.9

[1] Catechin content for each formulation determined by HPLC-ECD analysis of original GT powder as described in *Materials and Methods*. For *In vitro* digestions formulations were diluted in 15 mL double distilled water. For animal gavage solutions were dissolved in 1.5 mL physiological saline.

Table 2

Plasma pharmacokinetic parameters from Sprague Dawley rats gavaged with different GT formulations.[2,3,4]

	$AUC_{(0-6)}$ [pmol·h/L]	Tmax [hours]	Cmax [pmol/L]
EGC			
GT Only	1304.1±405.0 a	1.6±0.7 a	796.6±266.5 a
GT+Suc	2569.3±518.3 a,b	2.1±0.3 a	786.7±140.6 a
GT+AA	2775.1±438.1 a,b	1.4±0.2 a	1104.8±152.3 a
GT+Suc+AA	3237.0±466.1 b	1.7±0.2 a	1100.3±218.7 a
EC			
GT Only	2051.4±327.8 a	1.0±0.0 a	1023.2±201.9 a
GT+Suc	1389.1±134.8 b	1.9±0.1 b	459.7±69.8 b
GT+AA	1980.4±256.9 a	1.4±0.3 a	860.4±105.9 a
GT+Suc+AA	1365.7±181.6 b	2.0±0.5 b	433.6±50.8 b
EGCG			
GT Only	61.0±26.6 a	1.6±1.0 a	19.8±5.9 a
GT+Suc	155.0±58.2 a,b	1.9±0.1 a	58.3±24.8 a,b
GT+AA	92.2±14.9 a,b	1.0±0.2 a	34.7±4.4 a,b
GT+Suc+AA	181.8±17.0 b	1.9±0.5 a	69.4±8.2 b
ECG			
GT Only	31.8±14.3 a,b	1.6±0.6 a	10.8±4.1 a
GT+Suc	20.2±4.6 a,b	2.3±0.8 a	7.3±0.9 a
GT+AA	8.4±3.6 b	1.4±0.5 a	10.4±4.3 a
GT+Suc+AA	43.6±13.3 a	1.6±0.5 a	15.3±4.1 a

[2] AUC_{0-6hr} = Area under the plasma pharmacokinetic curve from 0 to 6h; C_{max} = Maximum plasma catechin concentration; T_{max} = Time of maximum plasma catechin concentration.

[3] Data is expressed as mean ±SEM.

[4] Presence of different letters indicates a significant ($P<0.05$) difference between plasma PK parameter between formulations for a specific catechin.