Effects of Infusion Time and Addition of Milk on Content and Absorption of Polyphenols from Black Tea

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Epidemiological studies assessing the health benefits of drinking black tea are equivocal. Such disparity may reflect an inability of semiquantitative assessment to consider how infusion time and addition of milk affect the bioavailability of potentially beneficial antioxidant polyphenols. Six brands of tea demonstrated similar increases in antioxidant capacity and total phenolic and catechin contents with increasing infusion time. These results were unaffected by the addition of milk. Consumption of black tea (400 mL) was associated with significant increases in plasma antioxidant capacity (10%) and concentrations of total phenols (20%), catechins (32%), and the flavonols quercetin (39%) and kaempferol (45%) (all $p < 0.01$) within 80 min. This was unaffected by adding milk. Infusion time may therefore be a more important determinant in the absorption of polyphenols from black tea. Observational studies assessing the health benefits of tea consumption require recording of brewing methods as well as frequency of consumption.

KEYWORDS: Black tea; polyphenols; flavonoids; catechins; antioxidant capacity; infusion time; absorption; milk

INTRODUCTION

Black tea consumption is associated with reduced risk of cardiovascular disease and several cancers (*1*). These beneficial effects have been ascribed to the marked antioxidant potential of polyphenolic compounds in the beverage. These are primarily flavan-3-ols (catechins), flavonols, theaflavins, and thearubigins (**Figure 1**) (*2, 3*), which chemical and animal models and human intervention trials (*1, 4*−*9*) suggest have anti-atherosclerotic and anticancer properties. However, the epidemiological data relating tea consumption and disease incidence are equivocal (*10*−*13*). This disparity may, in part, be due to the semiquantitative assessment of tea drinking habits in epidemiological studies. Often "number of cups of tea per day" is the only measure of tea consumption. Little consideration has been given to different brands and varieties of tea consumed or to methods of preparation, such as brewing time. Moreover, in the United Kingdom, milk is commonly added to black tea, which may lead to the formation of polyphenol−milk protein complexes. This may decrease the bioavailability and antioxidant potential of polyphenols in vivo (*14*−*18*), thus negating the anti-atherosclerotic benefit of black tea. In contrast, others report no masking effects of milk addition on increases in plasma antioxidant potential (*19*) and concentrations of catechins (*20*) or flavonols (*21*) after the consumption of black tea. However, interpretation of such studies is difficult as they consider different polyphenolic tea components and use antioxidant assay systems differing in choice of oxidation source, target, and type of measurement used (*2*, *22*, *23*).

Consequently, the current investigation considered two potential sources of variation in epidemiological assessment of tea consumption. First, the effect of infusion time on concentrations of the total phenols and the antioxidant potential of six commercial black teas was determined. The brand with the highest phenolic content and antioxidant capacity level was then used in a controlled intervention study with human volunteers to assess the effects of adding milk on antioxidant capacity and concentrations of total phenols, catechins, and flavonols in plasma.

MATERIALS, SUBJECTS, AND METHODS

Six different brands of black tea were purchased from a local U.K. retailer. Quercetin and kaempferol were purchased from Fluka (Gillingham, U.K.) All other reagents were obtained from Sigma-Aldrich (Poole, U.K.) or Merck (Poole, U.K.) unless specified.

Three grams of each brand of tea (equivalent to one U.K. tea bag) was infused for 3, 5, 7, and 10 min in 300 mL of freshly boiled water. Each tea infusion was stirred once when the water was added and filtered at the end of the infusion time to remove any residual tea leaves. Test infusions were quickly cooled on ice and snap frozen in liquid nitrogen before storage at −80 °C prior to analysis.

Nine healthy male volunteers (24−37 years of age) with a mean body mass index of 25 kg/m^2 (range = 18−35 kg/m^2) participated in the intervention trial. All subjects were normotensive, reported no

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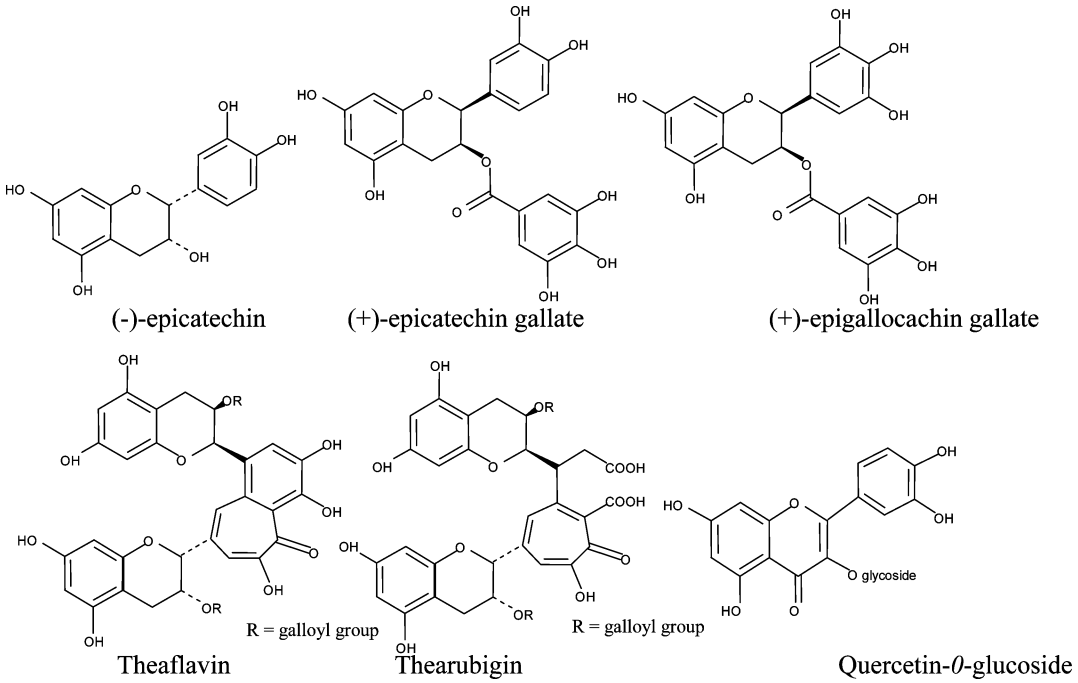

Figure 1. Chemical structures of the main flavonoids in black tea.

previous history of chronic disease, and had not taken any medication or nutritional supplements for at least 1 month prior to starting the trial. The Grampian Research Ethics Committee approved this study, and volunteers gave informed consent before participating. The trial followed a Latin square format where volunteers were randomly assigned to three groups and consumed three different beverages on three different occasions one week apart. After refraining from drinking tea for 24 h and fasting overnight, volunteers drank 400 mL of either black tea with water (3:1, v/v), black tea with milk (3:1, v/v), or a control beverage (water plus milk, 3:1, v/v). Three grams of tea was infused for 7 min in 300 mL of freshly boiled water. The infusion was filtered before the addition of 100 mL of either semiskimmed milk or cold water. Four hundred milliliters (two cups) of tea represents the average daily consumption, whereas semiskimmed cow's milk is the most common type of milk purchased in the United Kingdom (*24*). The control beverage consisted of 300 mL of freshly boiled water and 100 mL of semiskimmed milk. Concentrations of total phenols, catechins, and antioxidant capacity were also measured for each batch of test beverage produced, to ensure continuity between test days.

During each stage of the trial four venous blood samples were collected from each subject into vacutainers containing EDTA anticoagulant (Evacuette, Greiner, Labortechnik, Austria). Blood samples were drawn 10 min before the volunteer drank one of the test beverages, then 50, 80, and 180 min thereafter. Volunteers refrained from eating and drank no more than 100 mL of water—in addition to the test drink—until after collection of the final blood sample. Blood samples were stored on ice for a maximum of 30 min prior to centrifugation (4 °C, 1500*g*, 15 min). Plasma was then harvested, aliquoted, snap frozen in liquid nitrogen, and stored at −80 °C until analysis.

Phenolic and catechin compounds in tea and plasma were extracted by solid-phase extraction with aluminum oxide using a method described by Kivits et al. (*25*) with some modifications. In brief, 1 mL of plasma was vortex mixed with 3 mL of methanol [containing 1 g/L butylated hydroxytoluene (BHT)] for 5 min at 4 °C under nitrogen. The sample was then centrifuged (2000*g*, 15 min, 4 °C) before the supernatant was applied, under vacuum, to a preconditioned 1 mL alumina cartridge containing 100 mg of aluminum oxide (Waters Ltd.). The phenolic and catechin compounds present in the plasma-based supernatant readily bind to the aluminum oxide. Any residual unbound particles were removed by washing the cartridge with methanol and diethyl ether. The alumina-bound phenolic extract was subsequently eluted using 0.55 mL of methanol/percholic acid (60% vol)/water (8:1:1, v/v/v). All detectable compounds were eluted after one application of the elutant to the cartridge, because no phenolic compounds were found after a second wash of the cartridge. The collected extract was split into two aliquots for total catechin and phenolic measurements. A nitrogen-rich atmosphere was maintained throughout the extraction process. Total tea and plasma catechins were subsequently determined as described by Kivits et al. (*25*) with one adjustment: a 60 mmol solution of dimethylaminocinnamaldehyde (DMACA) was added to the test extract. Recovery of catechin-spiked plasma was 94 ± 2% (mean ± SEM), whereas inter- and intra-assay variations were 10 and 8%, respectively. Total tea and plasma phenolic compounds were measured according to the method of Swain and Hillis (*26*) as gallic acid equivalents. The mean (SEM) percentage recovery of gallic acid from a plasma spike was 96.3%, whereas inter- and intra-assay variations were 8 and 7%, respectively.

Tea and plasma flavonols were determined as aglycones of quercetin and kaempferol by fluorometric HPLC detection with postcolumn derivatization following deglycosylation with β-glucoronidase and acid hydrolysis (*27*). The total antioxidant capacities of tea and plasma samples were estimated using the ferric reducing ability of plasma (FRAP) assay (*28*).

Data are expressed as mean ± SD. Variables were checked for deviation from a normal distribution. Total plasma catechins and quercetin were skewed (skewness >1) and thus were transformed to natural logarithms for statistical analysis. The effect of treatment over time was initially assessed by single-factor ANOVA and two-sided *t* tests. Two-sided *p* values were considered to be significant at $p < 0.05$. Data were also analyzed by hierarchical ANOVA, with strata for subject, treatment within subject, and simple time within treatment. The effects of treatment, time, and a treatment and time interaction were assessed by standard *F* ratios. As a comparison between black tea with and without milk was of particular interest, a post-hoc assessment of this difference was made by repeating the ANOVA with the milk treatment omitted.

RESULTS

Different brands of tea exhibited similar infusion patterns for the release of phenolic and catechin compounds from tea leaves. Total phenol, total catechin, and antioxidant (FRAP) concentrations increased with infusion time, leveling off after 7 min (**Figures 2** and **3**). Tea infusions contained 87% of their final catechin concentrations within 3 min compared with only 60%

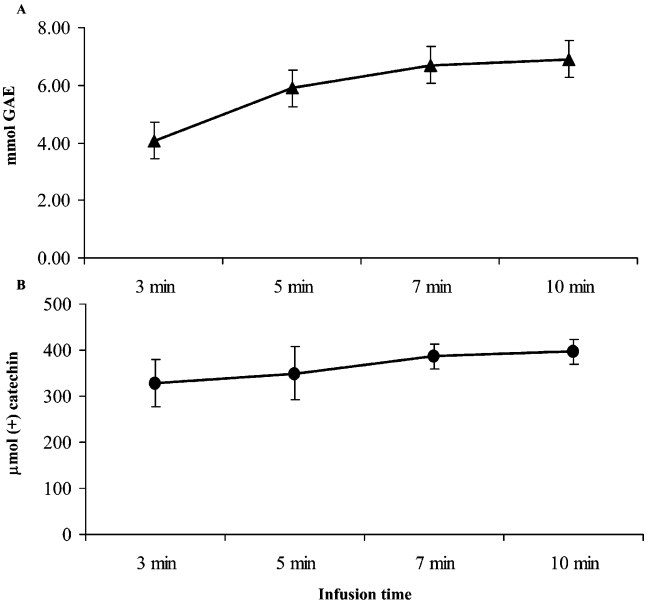

Figure 2. Effect of increasing infusion over time on levels of total phenols (▲) and total catechins (●) in black tea (3 g/300 mL of water).

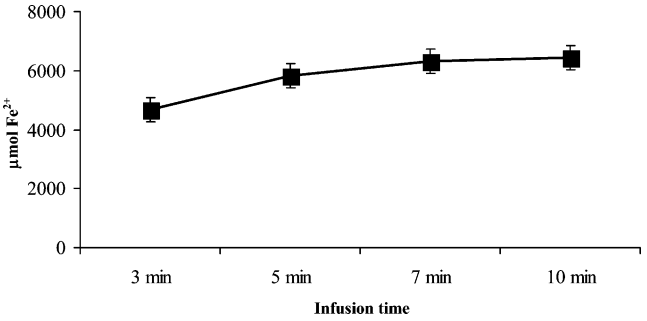

Figure 3. Effect of increasing infusion time on the development of antioxidant potential in black tea (3 g/300 mL).

Table 1. Total Phenol and Catechin Concentrations and Antioxidant Potential As Determined by FRAP Assay of Six Different Brands of Black Tea after a 7 min Infusion

	phenols		catechins		FRAP	
tea	mmol of GAE	±SD	μmol of catechin	±SD	μmol of Fe^{+}	±SD
1	6.87	0.06	420	1.03	6108	0.297
2[a]	3.55	0.02	340	4.65	4771	0.636
3	6.33	0.07	398	4.47	5305	0.958
4	7.78	0.03	379	2.72	7277	1.115
5	6.78	0.05	391	4.10	6210	0.460
6[b]	8.84	0.02	395	4.89	9064	0.265
mean	6.69	1.70	387	24.8	6468	1538

[a] Significantly different from other brands of tea ($p < 0.05$). [b] Tea consumed during human intervention trial.

for total phenols. Increases in FRAP value correlated with increases in both total phenols content ($r = 0.67$, $p < 0.001$) and total catechin content ($r = 0.80$, $p < 0.001$). No significant differences were observed between five of six brands of tea for total phenolics, catechins, and FRAP value after 7 min of infusion (**Table 1**). The addition of milk did not significantly affect concentrations of FRAP (**Figure 4**).

The brand of tea exhibiting the highest total phenolic and catechin contents as well as FRAP value (brand 6, see **Table 1**) was used in the human intervention trial. Six batches of test tea were prepared (3 g of tea leaves in 300 mL of water, infused

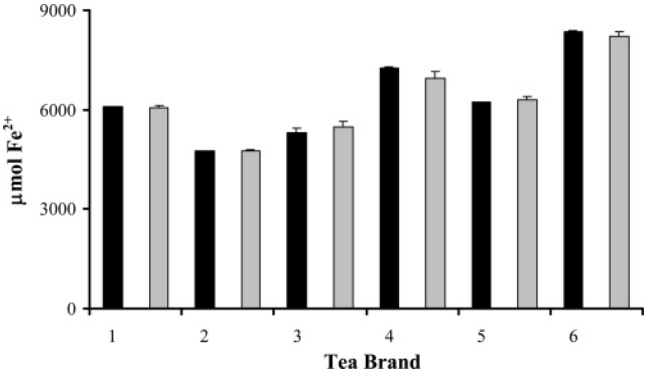

Figure 4. Effect of adding 100 mL of water (■) or semiskimmed milk (■) to tea infusions (3 g/300 mL).

for 7 min) during the course of the intervention trial. Total phenols, catechins, and FRAP levels were determined, and interbatch variations were 9.3, 4.4, and 7.2%, respectively. Flavonol determination of the test tea indicated higher levels of kaempferol than quercetin, 26.2 ± 9.70 and 2.40 ± 050 μmol, respectively. This finding was confirmed after the tea's flavonol composition had been profiled using mass spectrometry (personal communication, B. Mullen, University of Glasgow). The flavonol profiles indicated kaempferol compounds exhibited 50% higher signal intensities than quercetin compounds (**Figure 5**).

Drinking two cups of black tea resulted in significant transient increases of plasma total phenolic compounds ($p < 0.001$), total catechins ($p < 0.001$) (**Figure 6**), and the flavonols quercetin ($p < 0.01$) and kaempferol ($p < 0.001$) (**Figure 7**). During the 3 h time course total phenolic, quercetin, and kaempferol levels were highest 50 min postingestion with 20, 30, and 45% increases from baseline concentrations of 1.59 ± 0.08 mmol GAE, 11.26 ± 0.99 nmol, and 40.27 ± 2.77 nmol, respectively. Total catechin concentrations peaked after 80 min with average increases of 32% from the baseline of 0.79 ± 0.04 μmol. Magnitudes of response for total plasma phenolics, catechins, and quercetin were unaffected by the addition of milk to tea, with no significant differences between treatments. Addition of milk to tea weakly, but not significantly ($p < 0.07$), affected plasma kaempferol response to black tea ingestion (**Figure 7**). Drinking the control beverage produced no significant changes in plasma phenol, catechin, or quercetin concentrations, whereas kaempferol levels fell significantly ($p < 0.05$).

Consuming the control beverage did not significantly change baseline antioxidant levels (FRAP), 865.0 ± 53.93 μmol of Fe^{2+}/L, during the 3 h postingestion period. Conversely, drinking black tea on average increased the antioxidant potential of plasma by 10% ($p < 0.01$) within 80 min (**Figure 8**). The 7% ($p < 0.05$) increase observed when milk was added to the beverage was not significantly different from the response following the consumption of black tea.

DISCUSSION

In agreement with previous findings, black tea proved to be a rich source of polyphenolic compounds with strong in vitro antioxidant potential (*21*, *29*−*31*). The observed effects of increasing total phenols and antioxidant concentrations with increasing infusion time are also known to be influenced by stirring duration and intensity, leaf size, and tea bag porosity (*30*, *32*). Taken together, these observations re-emphasize the importance of gathering brewing and preparation details in addition to numbers of cups consumed when associations

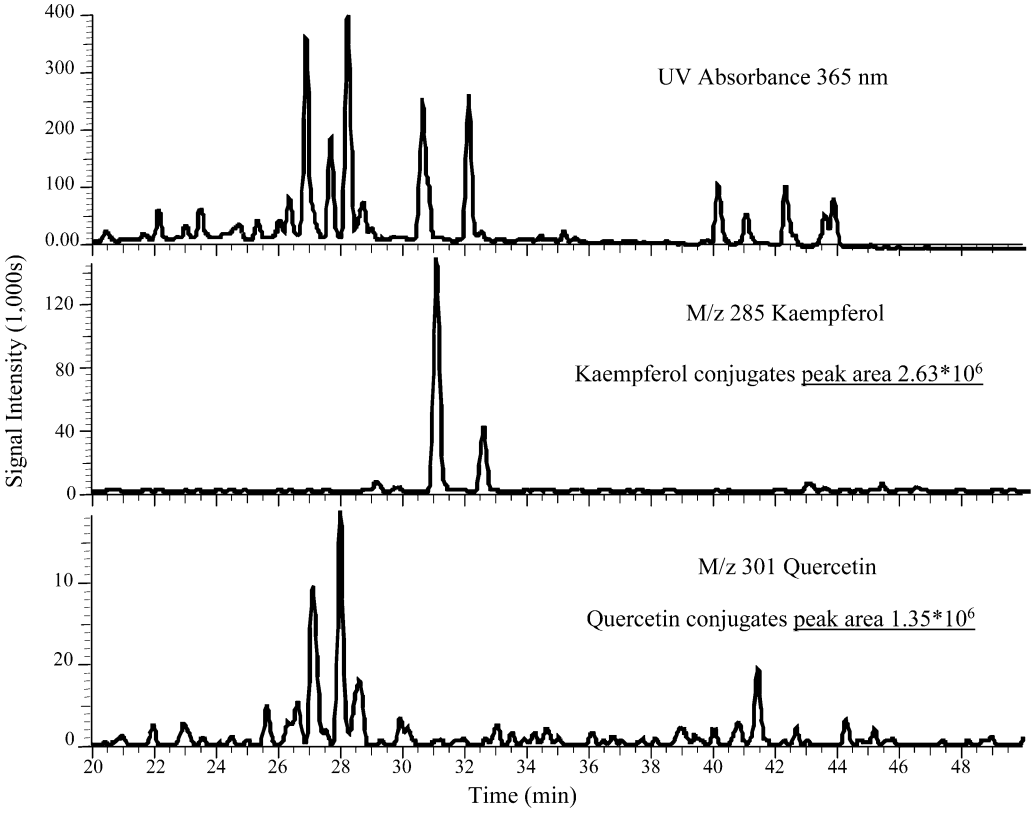

Figure 5. Flavonol characterization of tea infusion using mass spectrometry.

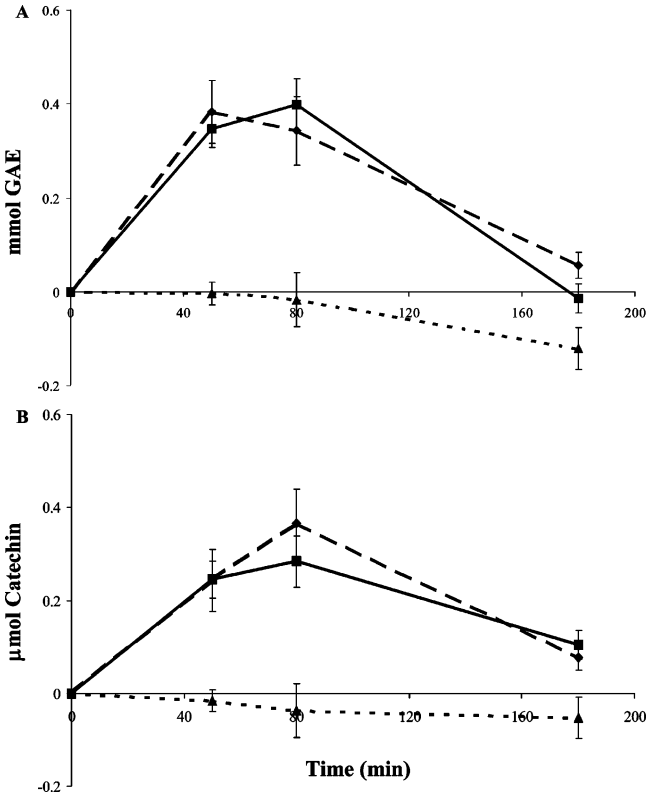

Figure 6. Change in plasma concentrations of (**A**) total phenols (expressed as gallic acid equivalents) and (**B**) catechins over 180 min after drinking (400 mL) of black tea (◆), black tea with milk (■), and water with milk (▲) (mean average from zero ± SEM).

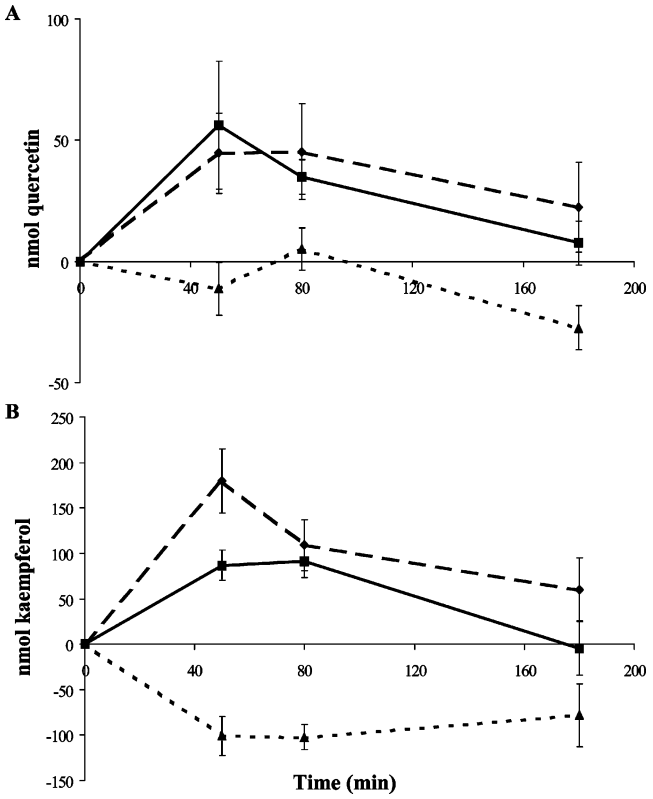

Figure 7. Change in plasma (**A**) quercetin and (**B**) kaempferol concentrations with time after drinking (400 mL) of black tea (◆), black tea with milk (■), and water with milk (▲) (mean average from zero ± SEM).

between tea and disease risk are tested. The addition of milk did not decrease the in vitro antioxidant potential of tea, which is in agreement with one previous study (*17*), but in contrast with another (*14*). Our results suggest that the formation of milk protein−polyphenol complexes does not compromise the antioxidant potential of the beverage.

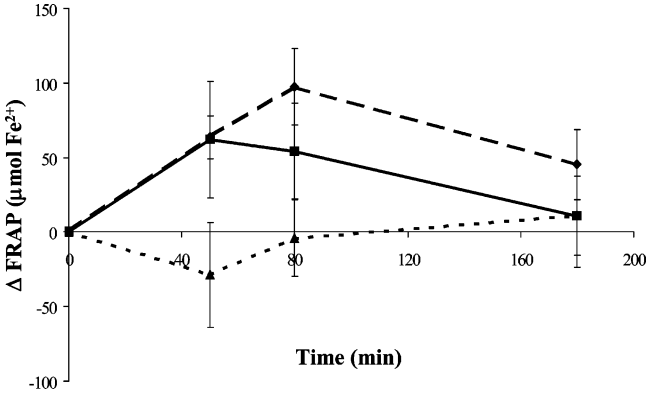

Figure 8. Change in total antioxidant capacity (FRAP) of plasma over 180 min after drinking (400 mL) of black tea (◆), black tea with milk (■), and water with milk (▲) (mean average from zero ± SEM).

At the time of writing, this was the only controlled intervention study to assess changes in antioxidant capacity in conjunction with plasma total phenols, total catechins, and flavonols after the consumption of tea with and without milk. This approach was primarily aimed at clarifying the contradictory evidence of the effects of adding milk to tea on the uptake of antioxidant phenolic compounds.

The absence of an inhibitory affect of milk on the uptake of tea catechins supports the findings of van het Hof and colleagues (*20*). Average maximum increases of plasma catechin concentrations (0.37 μmol/L) after the consumption of black tea were also within the expected response range (*9*). However, basal plasma levels of total catechins (0.72 ± 0.12 μmol of catechin/L) and total phenols (1.55 ± 0.07 mmol of GAE) in the present study were higher than previously reported, 0.06 ± 0.03 μmol/L (*25*, *33*) and 0.95−1.00 mmol of GAE (*22*, *34*), respectively. These higher baseline values may be due to the modified extraction method introduced here. The original, manually packed, solid-phase extraction method (*25*) showed considerable variation in the recovery of catechins from spiked plasma with interassay variation being around 30%. After prepacked Sep-Pak alumina cartridges (Waters, Poole, U.K.) were introduced, catechin recovery improved to 94%, compared to 78% reported by Kivits et al. (*25*), and interassay variation was also reduced to 10%. Furthermore, the use of sealed cartridges permitted collection of an alumina-free extract as well as facilitating fractionation of the extract to measure both total catechins and total phenols.

Measurement of total phenolic uptake into plasma after drinking tea by the Folin−Ciocalteu method, although relatively nonspecific, provided an indication of the presence of oxidizable phenolic groups containing a benzene ring. Therefore, compounds including gallic acid, flavonoids, condensed flavonoids such as theaflavins and thearubigins, and semiquinones can be quantified (*35*). This is particularly important when individual characterization of 58−75% of tea phenolics remains undefined (*2*, *23*). More specifically, formation of the color complex between catechins and DMACA enabled measurement of compounds containing meta-oriented hydroxy groups in the A-ring of the flavane with a single bond in the 2−3-position of the heterocyclic ring. Kivits and colleagues (*25*) found that catechin esters, including gallated esters, responded as strongly with DMACA as catechin. More complex condensed catechins, that is, theaflavins and thearubigins, only weakly complex with DMACA. Flavonols possessing a double bond at the 2−3-position of the C-ring do not complex with DMACA but do so with Folin−Ciocalteu reagent.

Uptake of tea flavonols in the present study resulted in average maximum increases in plasma levels of 4.5 and 17.9 nmol, respectively. Similar increases have been observed after the ingestion of quercetin- and kaemperol-rich foods (*36*). The greater increase of plasma kaempferol reflects the unusually high levels of kaempferol compared to quercetin glycosides of the test tea. Typically, quercetin glycosides were thought to be the dominant flavonols present in black tea (*23*, *37*). The addition of milk to the beverage did not suppress flavonol uptake, supporting previous findings (*21*). Pre-intervention levels of quercetin reported here were comparable with previously reported concentrations—ranging from 0 to 20 nM (*38*, *39*). However, the basal plasma kaempferol levels of 38−49 nM observed in this study were greater than those previously reported (approximately 5 nM) (*21*, *36*).

The lack of suppressive effects of adding milk to tea on phenolic uptake is reflected by analogous increases in plasma antioxidant capacity. This suggests that some of the tea polyphenols not only are bioavailable but also retain hydroxyl groups capable of hydrogen donation in vivo. Langley-Evans (*18*) reported that drinking tea with milk had a suppressive effect on increases of antioxidant capacity of whole blood compared with drinking tea alone. However, this study (*18*) recorded very high FRAP values, suggesting that comparison of antioxidant capacity between plasma and whole blood is problematical. A number of human intervention studies have also recorded increases in plasma antioxidant potential in response to black tea consumption ranging from 2 to 52% (*8*). Differences in study design, brand of tea, type of milk, and assay system used may account for such variation. However, Leenen and colleagues (*19*) using the FRAP assay reported findings similar to that obtained in the present study. In addition, drinking the tea with full-fat milk did not affect changes in plasma antioxidant capacity.

By demonstrating the effects of increasing infusion time on phenolic content and antioxidant capacity of tea, the current investigation has highlighted the importance of collecting brewing details when relationships between tea consumption and disease risk are assessed. Moreover, the addition of milk to black tea should not confound epidemiological studies as increases in plasma antioxidant capacity, catechins, and flavonols are not adversely affected.

ACKNOWLEDGMENT

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