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Effect of tea phenolics on iron uptake from different fortificants by Caco-2 cells

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ABSTRACT

The *in vitro* effects of tea phenolics on Fe uptake from different fortificants ($FeSO_4$, $FeCl_3$, FeEDTA) by Caco-2 cells were compared. Cell cultures were exposed to catechin, tannic acid, green or black tea solutions, added within Fe-containing solution, or used to pre-treat cell cultures before Fe-exposure. Cell ferritin formation was used as a measure of Fe uptake. Reverse phase chromatography was used to identify specific phenolics in tea solutions, and the Fe-binding catechol and galloyl groups were determined spectrophotometrically. The results showed a positive effect of catechin on Fe uptake only from dissociable Fe sources, and a marked inhibitory effect of tannic acid regardless of the Fe source. Tea phenolics exhibit similar inhibitory patterns on Fe uptake from $FeCl_3$ and FeEDTA solutions; however, the Fe uptake from $FeSO_4$ solutions was significantly less affected. These data improve the understanding of interactions by which tea phenolics affect Fe uptake at the intestinal level.

1. Introduction

Iron (Fe) deficiency is a leading nutritional concern worldwide and, as such, is the most prevalent mineral deficiency (Beard & Stoltzfus, 2001). It is well known that Fe availability is dependent on the chemical form of Fe, and it is affected by interactions with various dietary components that can impair or improve non-heme Fe absorption from foods (Powell, Whitehead, Lee, & Thompson, 1994). The addition of tea and/or its extracts to foods has been related to decreased non-heme iron absorption in animals (Valdez, Gee, Fairweather-Tait, & Johnson, 1992) and humans (Merhav, Amitai, Palti, & Godfrey, 1985; Samman et al., 2001) studies.

Tea constitutes the most commonly consumed beverage in the world, after water (Benzie & Szeto, 1999). Tea is prepared from dried leaves of *Camellia sinensis* and can be classified mainly into green and black tea (Belitz & Grosch, 1999). Black tea pigments are thought to be formed by the enzyme-catalysed oxidation of the catechins of green tea (Bailey & Nursten, 1993). It is among the many plant products that provide flavonoids to the human diet – phenolic compounds make up 25–35% of the dry matter content of young, fresh tea leaves (Belitz & Grosch, 1999). Phenolic contents can vary under field conditions, and according to seasonal, genetic and agronomic factors (Yao et al., 2005). Polyphenolic compounds are reported to exhibit antioxidant activity and could confer positive health benefits on humans (Liu, 2003). Tea (*C. sinensis*), due to its phenolics content, is reported to have antioxidant activity *in vitro* (Galati, Lin, Sultan, & O'brien, 2006). Previous studies showed that epigallocatechin, epicatechin gallate, and epigallocatechin gallate were the main flavonols in tea (*C. sinensis* var.) (Belitz & Grosch, 1999; Samman et al., 2001). The antioxidant abilities of these chemical structures vary as follows: epicatechin gallate > epigallocatechin gallate > epigallocatechin > gallic acid > epicatechin = catechin (Salah et al., 1995). In addition, tannins, which are naturally occurring plant phenols, have also been recognized as antioxidants but cytostatic and cytotoxic effects have been attributed to the hydrolysable fraction (Labieniec & Gabryelak, 2003).

While the concept that polyphenols can provide positive health effects is gaining acceptance, there is a paucity of information regarding the mechanisms through which these compounds act. Recent reports have shown that antioxidant action is dependent on the ability of phenolics to scavenge free radicals and/or to chelate metals (Azam, Hadi, Khan, & Hadi, 2004; Galati et al., 2006; Zhu, Lazarus, Holt, Orozco, & Keen, 2002). It was suggested that Fe binds to polyphenols via the ortho dihydroxy (catechol) or trihydroxy-benzene (galloyl) group (Brune, Hallberg, & Skånberg, 1991; Khokhar & Apenten, 2003). Otherwise, tannins can also produce complexes with Fe^{+2} [$(Fe^{+2})_n$-tannic acid] (Lopes, Schulman, & Hermes-Lima, 1999). In addition to their metal chelating ability, the antioxidant effects could also be derived from their different hydro-philicity/phobicity which condition their interaction with the biological membranes, increasing their fluidity (Erlejman, Verstraeten, Fraga, & Oteiza, 2004; Verstraeten, Keen, Schimtz, Fraga, & Oteiza, 2003).

In recent years, many efforts have been made to develop methods to accurately evaluate Fe availability from foods and diets. The *in vitro* methods provide an effective approximation to the *in vivo* situation. A Caco-2 cell culture model has demonstrated strong

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correlations with human studies in predicting the intestinal response to enhancers and inhibitors of Fe absorption (Yun, Habicht, Miller, & Glahn, 2004). Furthermore, the Caco-2 cells model has proved a valuable tool for predicting the correct direction of the intestinal response to inhibitors of Fe absorption (Fairweather-Tait et al., 2005). This cell culture model constitutes a valuable tool for improving the understanding of the mechanism(s) involved on Fe absorption at intestinal level (Johnson, Yamaji, Tennant, Srai, & Sharp, 2005).

The aim of the present study was to evaluate the effects of phenolics found in tea on iron availability of common sources of fortificant iron, using Caco-2 cells. This study was designed to compare the effects of phenolics in tea when those were added jointly within Fe-containing solutions or after pretreating cell cultures with different phenolics (catechin or tannic acid) or tea (green or black) solutions. The most widespread non-heme Fe sources, used as fortificants, include, among others, FeEDTA and $FeSO_4$ (Huma, Rehman, Anjum, Murtaza, & Sheiki, 2007). In addition, a dissociable ferric salt ($FeCl_3$) was also compared.

2. Material and methods

2.1. Reagents

Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). All glassware used in the sample preparation and analyses was treated with 10% (v/v) HCl, concentrated (37%) for 24 h, and then rinsed with 18 MΩ cm deionised water before being used.

2.2. Instruments

An inductively coupled argon plasma emission spectrometer (ICP-ES, Model 61E Trace Analyzer, Thermo Jarrell Ash Corporation, Franklin, MA), a spectrophotometer (DU 520 UV/vis, Beckman Coulter, BC), and an automatic gamma counter Wizard 3 Wallac 1480 (Perkin Elmer, USA) were used. For phenolics speciation analysis, the HPLC system employed (Waters, Milford, MA, USA) was equipped with a 600E multisolvent pump, a 717 plus auto sampler and a 996 photodiode array detector set at 264 nm.

2.3. Iron standards and working solutions

Several iron standards, such as ferrous sulphate ($FeSO_4$) (Mallinckrodt AR©, Germany), ethylenediaminetetraacetate, (NaFeEDTA) (EDFS, Sigma) or Ferric chloride ($FeCl_3$) (High Purity Standards, Cat. 100026-2), were used. Working solutions were prepared by dissolving appropriate amounts of the standards in 0.1% HCl aqueous solutions.

2.4. Tea preparation

Commercial ready-to-use samples of green and black tea (bags) were obtained from food stores in Ithaca, New York (USA). Aliquots (2.20 ± 0.07 g) of each, green and black tea, were soaked in 100 ml of deionised water and boiled for 15 min in a container covered by a watch glass to create a reflux system and minimize loss of water. After cooling to room temperature, aliquots of the mixture were transferred to polypropylene centrifuge tubes and centrifuged (2057 g/10 min/4 °C) to separate the soluble fraction, which was pooled for each tea. These obtained fractions were kept at −20 °C until used.

2.5. Total phenolic content in tea solutions

The amount of total phenolics was determined using the Folin-Ciocalteau method (Dewanto, Wu, Adom, & Liu, 2002). Briefly, to 125 μl of the solution from boiled tea, 500 μl of deionised water and 125 μl of the Folin-Ciocalteau reagent were added. The mixture was allowed to stand for 5 min, and then, 125 μl of a 7% aqueous Na_2CO_3 solution were added. The final volume of the mixture was adjusted to 3 ml with deionised water and it was allowed to stand for 60 min at room temperature. The absorbance was measured at 760 nm against a reagent blank. The amount of total phenolics was expressed as gallic acid (G7384, Sigma) (mg g^{-1} of sample). A calibration curve of gallic acid (ranging from 2–10 μg ml^{-1}) was prepared and the results, determined from a regression equation of the calibration curve, were expressed as mg gallic acid per gramme of the tea sample.

2.6. Iron-binding phenolic groups in tea solutions

The total galloyl and catechol groups, which react with iron, were determined spectrophotometrically (Brune et al., 1991). To 2 ml of the tea solution, 8 ml of dimethylformamide (DMF)-0.1 M acetate buffer (pH 4.4) mixture (50% v/v) were added. An aliquot of 2 ml of the previous prepared solution was placed in a 10 ml tube and 8 ml of fresh FAS-reagent (89 parts of 50% w/v urea-0.1 M acetate buffer, 10 parts of 1% gum Arabic solution, and 1 part of 5% ferric ammonium sulphate dissolved in 1 M HCl) were added. After 15 min, the absorbances at 578 nm (galloyl groups) and 680 nm (catechol groups) were read versus a reagent blank consisting of 2 ml DMF-acetate and 8 ml FAS-reagent. The content of galloyl and catechol groups in the sample was, therefore, calculated using linear regression equations for the standard curves prepared using catechin (C-1251, Sigma) (catechol groups) and tannic acid (T8406, Sigma) (galloyl groups).

2.7. HPLC analysis of flavonoids in tea solutions

Flavonoids were analysed according to a previously described method (Espinosa-Alonso, Lygin, Widholm, Valverde, & Paredes-Lopez, 2006) with slight modifications. The analysis was carried out on HPLC system operated using Empower software. The separation was performed on a Vydac 5u 300A C18 column (Phenomenex) 250 × 4.6 mm. The gradient programme started with 100% of solvent A (1% acetronitrile in 20 mM phosphate buffer adjusted to pH 2.20 with concentrated H_3PO_4), and solvent B (70:30 v/v, water:acetronitrile; HPLC grade) was increased linearly to reach 10% in 2.5 min. From 2.5 to 6 min, the flow was increased linearly to 12% of solvent B. From 6 to 16 min the flow was increased linearly to 23% of solvent B. From 16 to 22 min the flow was increased linearly to 35% of solvent B. From 22 to 24 min, the flow was increased linearly to 95% of solvent B. From 24 to 30 min, the flow rate was maintained constant at 95% of solvent B. The column was equilibrated, using the initial conditions, for 10 min. UV absorbance at 260 nm was used to detect flavonoids. Total running time for each analysis was 30 minutes. Tea solutions were filtered through 0.45 μm (Nylon filters, Fisher brand), and adequate dilutions were prepared by using deionised water (18 MΩ cm). Then, 150 μl of the filtrates were injected into the HPLC system.

2.8. Cell cultures

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17 and used in experiments at passage 25–33. Cells were seeded at a density of 50,000 cells cm^{-2} in collagen-treated six-well plates (Costar, Cambridge, MA, USA) and maintained with Dulbecco's modified Eagle's medium (DMEM) under conditions previously described (Glahn, Lee, Yeung, Goldman, & Miller, 1998). The cells were used for iron uptake experiments at 13 days post seeding. On the day prior to the *in vitro* digestion experiment, the DMEM medium was removed