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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

and washed with 2 ml of MEM, and then the cells, with another 2 ml of MEM, were returned to the incubator.

2.9. In vitro digestion

To simulate gastrointestinal digestion, a previously described method was applied (Glahn et al., 1998). Porcine pepsin (P-7000) (800–2500 units/mg protein), pancreatin (P1750) (activity, 4×USP specifications) and bile extract (B8631) (glycine and taurine conjugates of hyodeoxycholic and other bile salts) were demineralised with Chelex-100 (Bio-Rad Laboratories, Hercules, CA, USA) before use.

Briefly, aliquots of catechin or tannic acid standard solutions, and green or black tea, all of them providing equal total phenolic contents, 20 or 200 μg gallic acid equivalents, were mixed with saline solution (140 mM NaCl, 5 mM KCl). Afterwards, the mixtures were treated, or not, with the Fe working solution (final amount of 20 μg as Fe). After the gastric stage (pepsin, pH 2 / 1 h / 37 °C), the intestinal phase (pancreatin-bile extract pH 7.0 / 2 h / 37 °C) of digestion was performed, with gentle rocking, in the upper chamber of a bicameral chamber system designed to separate the "gastrointestinal digest" from the Caco-2 cell monolayer. A 15,000 Da molecular weight cutoff dialysis membrane attached to a plastic insert ring was used to create the two chambers. Then the insert and digests were removed. An additional 1 ml of minimal essential medium (MEM) (Gibco) was added to the cell culture, and the system returned to the incubator for an additional 22 h. The next day, the cells from each well were washed twice with the isotonic saline solution and harvested in 2 ml of deionised-distilled water. Control solutions containing digestive enzymes or tea solutions (those volumes added to working solutions without Fe) were used in parallel. Cell ferritin formation was used as a measure of cell iron uptake.

2.10. Pre-treatment of cell cultures with phenolics

To evaluate the effect of phenolics on Fe uptake and their potential interaction within the brush border membrane of Caco-2 cells, different phenolics-containing solutions were prepared. Appropriate volumes from catechin or tannic acid standard solutions, and green or black tea solutions which provided the same total phenolic content, 20 or 200 μg gallic acid equivalents, were mixed in 15 ml of Hank's balanced salt solution (HBSS, Gibco). Then, 2 ml of the prepared solution were added to the cell cultures. Cell cultures were divided into two sets. In one of them, Caco-2 cells were pre-treated with phenolic-containing solutions without Fe. Afterwards, the cultures were returned to the incubator and incubated for 2 h (37 °C/5% CO_2 in a rocking shaker). After incubation, the culture medium was removed and replaced with 1 ml of fresh MEM. These cultures were exposed to gastrointestinal digests of iron-containing (20 μg as Fe) solutions, as described above. The other set of cultures were exposed to *in vitro* digests of the same phenolics solutions treated with Fe (final amount of 20 μg as Fe).

2.11. Ferritin and total protein assays

Caco-2 cell ferritin assays were performed with a one-stage, two-site immunoradiometric assay (FERIRON II ferritin assay, RAMCO Laboratories, Houston, TX, USA). A (10 μl) sample of each harvested cell culture was used for ferritin determination. A positive control (50 μM of iron with 500 μM ascorbic acid) was used to verify responsiveness of the Caco-2 cells to available Fe. The ferritin formation was expressed per unit of cell protein (ng mg^{-1} of protein). Caco-2 cell protein was determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The absorbance of the samples was measured at 750 nm. The experiments were conducted in triplicate on two different days.

2.12. Mitochondrial enzyme activities

These activities were evaluated by monitoring MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) conversion on exposed cultures after an incubation period (Laparra, Tako, Glahn, & Miller, 2008a, b).

2.13. Total iron determination

Fe concentration in all the prepared Fe-containing solutions was determined by ICP-ES after wet-ashing digestion (Laparra, Tako, Glahn, & Miller, 2008a, b). The instrument was calibrated with 10% $HClO_4$ as the low standard and 1 μg g^{-1} of Fe in a multi-element standard as the high standard. Fe was analysed using the 238.2 nm line.

2.14. Statistical analysis

A one-factor analysis of variance (ANOVA) and the Tukey test (Box, Hunter, & Hunter, 1978) were applied to determine statistical differences on ferritin, total phenols and phytate contents in analysed cultures and digests. A significance level of $p < 0.05$ was adopted for all comparisons. Statgraphics Plus version 5.0 (Rockville, Maryland, USA) was used for the statistical analysis.

3. Results and discussion

3.1. Iron concentrations

The Fe concentrations in the green and black tea solutions were 140 ± 30 and 134 ± 2 ng ml^{-1}, respectively. For the assays, Fe-containing solutions were freshly prepared before use and no significant ($p > 0.05$) differences in Fe concentrations among solutions prepared on different days were noted. Total Fe concentration in the solutions loaded into the upper chamber of the *in vitro* system, containing $FeSO_4$, $FeCl_3$ or FeEDTA were 21.2 ± 1.1, 20.9 ± 1.9 and 20.8 ± 2.0 μM as Fe (values obtained with $n = 6$, triplicate analysis of two independent prepared Fe-containing solutions), respectively. These concentrations were chosen from previous research of our group where Fe-deficient Caco-2 cultures have been shown to uptake, completely, the Fe content that diffused into the bottom chamber of the *in vitro* system (Glahn et al., 1998; Laparra et al., 2008a, b).

3.2. Polyphenols in green and black tea solutions

The total phenolics content (equivalents of gallic acid), catechol and galloyl Fe-binding groups (catechin and tannic acid equivalents, respectively), and the main polyphenols quantified in both green and black tea solutions are shown in Table 1. The structures of the catechins considered in this study are shown in Fig. 1. Tea solutions had a well-defined polyphenol composition and a fraction of tannins. It is important to mention the significantly ($p < 0.05$) lower content of catechin and epigallocatechin-3-gallate in black tea than in green tea. Otherwise, no statistical ($p > 0.05$) variation in epicatechin gallate content was quantified. Although, the naturally-occurring tannin fraction makes a major contribution to the total phenol content we were unable to quantify it by HPLC due to the non-linear response of the chromatographic signal. When considering the total galloyl groups present in both tea solutions (Brune et al., 1991), a higher ($p < 0.05$) content was estimated in black tea than in green tea solution. Despite the difference in

Table 1
Polyphenol content of the green and black tea solutions. Values are expressed as means ± standard deviation ($n = 3$). Different superscripts indicate ($p < 0.05$) differences between contents of same compounds in green and black tea.

Tea	Total (mg gallic acid/g)	Catechol groups (mg catechin/g)	Galloyl groups (mg tannic acid/g)	Compound (mg/ml solution)	
Green	236 ± 11.7[a]	18.2 ± 1.3[a]	74.7 ± 4.1[a]	Catechin	4.45 ± 0.14[a]
				Epicatechin gallate	2.56 ± 0.09[a]
				Epigallocatechin-3-gallate	7.77 ± 0.50[a]
				Tannins	NQ
Black	248 ± 2.6[a]	18.9 ± 0.7[a]	88.3 ± 1.3[b]	Catechin	1.03 ± 0.05[b]
				Epicatechin gallate	2.47 ± 0.22[a]
				Epigallocatechin-3-gallate	0.52 ± 0.05[b]
				Tannins	NQ

NQ, not quantifyable.

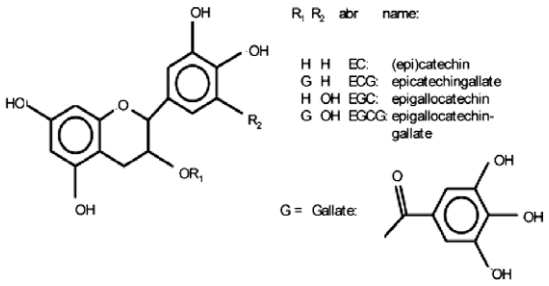

Fig. 1. Structures for some of the catechins found in green and black tea solutions.

galloyl groups, these solutions had similar ($p > 0.05$) total phenolics contents.

These results are concordant with previous reports that showed that epigallocatechin, epicatechin gallate and epigallocatechin gallate were the main flavonols in tea (*C. sinensis* var.) (Belitz & Grosch, 1999; Samman et al., 2001). Currently, it is accepted that polyphenols and hydrolysable tannins form insoluble complexes (Khokhar & Apenten, 2003) that can inhibit Fe absorption in the gut (Powell et al., 1994; Samman et al., 2001). These aspects are discussed below.

3.3. Effects of catechin and tannic acid on Fe uptake

After the uptake incubation period, the MTT conversion percentages obtained ranged from 87–103% with respect to the controls. These data provide evidence that energetic cell metabolism was not impaired and cell viability was adequate during all of the experiments. The effects of catechin and tannic acid standard solutions on Fe ($FeSO_4$, $FeCl_3$ and FeEDTA solutions) uptake by Caco-2 cells are shown in Figs. 2, 3, and 4A. The order of Fe uptake from the Fe sources evaluated was FeEDTA > FeSO4 > $FeCl_3$.

When considering the joint addition of catechin within the Fe-containing solution, through *in vitro* digestion, a positive effect on Fe absorption was noted. The latter seemed to be dose-dependent in the concentration range assayed. However, when cultures were pre-treated with catechin solutions, before being exposed to Fe-containing solutions, the Fe uptakes were similar ($p > 0.05$) for both concentrations assayed. Tannic acid solutions had a negative effect on Fe uptake with both ways of exposure. For the lower concentration assayed, this negative effect was markedly higher when cell cultures were pre-treated. Fe absorption was negligible from solutions of the highest concentration of tannic acid used.

In cultures exposed to $FeCl_3$ solutions, the combined presence of catechin and Fe, through *in vitro* digestion, also had a positive effect on Fe uptake. This observation followed the same pattern as that observed for $FeSO_4$ solutions. In $FeSO_4$ solutions, tannic acid exhibited a dose-dependent negative effect on Fe uptake. Interestingly, no differences of Fe uptake, among joint additions and pre-treatments of cell cultures, were noted with the lower concentration assayed. The highest concentration assayed completely abolished the Fe uptake.

A different pattern (from those described above) emerged when using FeEDTA solutions, a complexed and not easily dissociable source of Fe. Catechin had no effect on Fe uptake (in any of both ways of exposure to cell cultures) compared to FeEDTA exposure alone. Besides, tannic acid caused a significant ($p < 0.05$) decrease of Fe uptake when it was added jointly, within the Fe solution, reducing Fe uptake by 76.8% relative to the controls when cell cultures were pre-treated with the lowest concentration assayed. The latter negative effects on Fe uptake were markedly increased with the highest concentration of tea assayed.

Previous studies have shown that Fe binds to polyphenols via the ortho dihydroxy (catechol) or trihydroxy-benzene (galloyl) group (Brune et al., 1991; Khokhar & Apenten, 2003). Thus, the present study might have anticipated decreased Fe uptake derived from the soluble Fe-chelating ability of catechins in *in vitro* media. However, the increased ferritin values in $FeSO_4$- and $FeCl_3$-exposed cultures suggest that a standard solution of catechin (Sigma) had no inhibitory effect on Fe uptake by Caco-2 cells. In addition, it has been reported that flavanols (catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate) can interact with the polar head groups of phospholipids in the membrane surface of liposomes, thus decreasing membrane fluidity and surface potential (Erlejman et al., 2004; Tsuchiya, 1999; Verstraeten et al., 2003). From this scenario, we can speculate that distribution of flavanols within the hydrophobic phase of membranes may contribute to the cellular internalisation of bound Fe (from ionisable forms) and, in this way, favour Fe uptake, which would explain the effect noted. Besides, it seems that this effect is not dose-dependent. Besides, catechin evidently had no effect on Fe absorption from FeEDTA solutions. These results (Fig. 4A) agree with the same ($p > 0.05$) percentages of absorption for FeEDTA as previously reported, when it was given alone or with vegetable foods (Grebe, Foradori, Navarrete, Burgos, & Lira, 1979). The latter study suggested that FeEDTA was not affected by the ligands present in vegetable foods.

The non-alteration of Fe absorption relative to the independent exposure to the dissociable Fe-containing solutions, when cultures were pre-treated with catechin solutions before being exposed to the Fe-containing solutions, would confirm the Fe-binding ability of catechin and its participation in enhancing Fe uptake by Caco-2 cells. When pretreating the cultures, the partition of catechin in the hydrophobic phase of the biological membrane would not allow its interaction with soluble Fe in favouring Fe absorption, as it previously did when added jointly, within Fe-containing solutions. This observation could explain, at least in part, the results obtained with tea solutions (*see next section*). It has been reported that flavanols exhibit differences in their partition coefficients as a function of their structure – hydrophobicity: epicatechin gallate > epigallocatechin gallate > catechin (Erlejman et al., 2004).

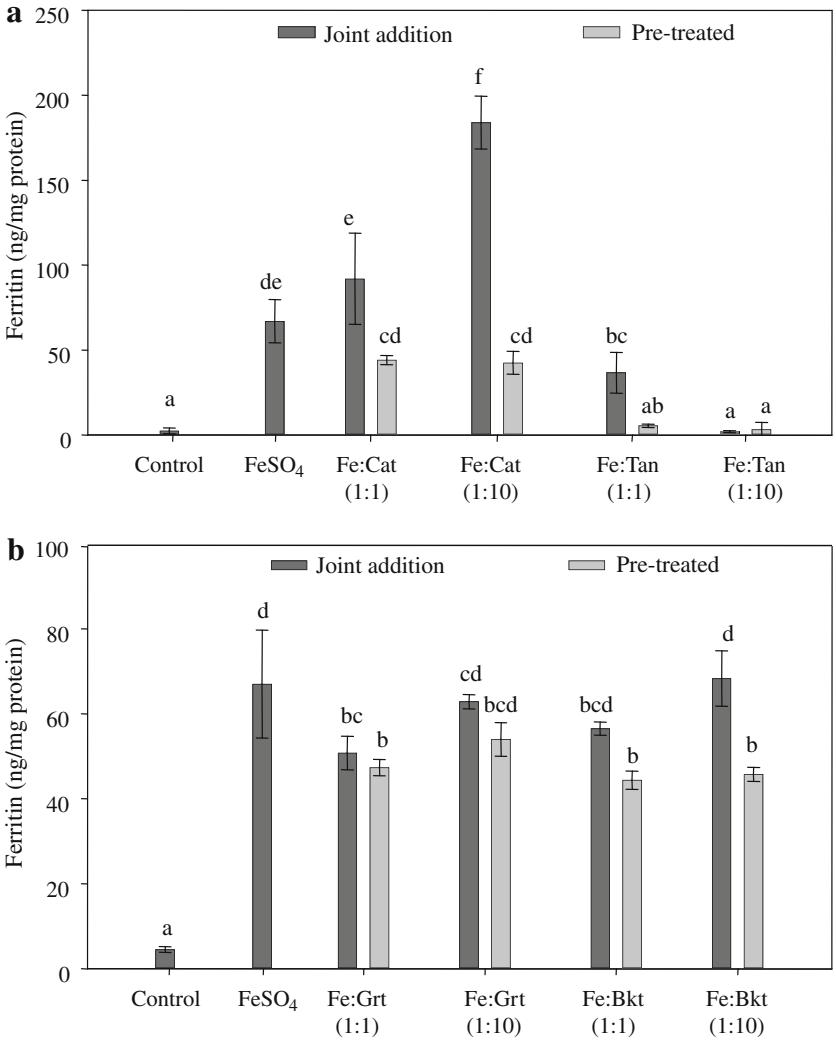

Fig. 2. Iron (Fe) uptake from $FeSO_4$ solution: (A) effect of catechin (Cat) or tannic acid (Tan), added during the digestion process or pretreating of the cultures (2 h), on Fe uptake; (B) effect of green (Grt) and black (Bkt) tea, added during digestion process or pretreating of the cultures (2 h), on Fe uptake. Values are expressed as means ± standard deviation ($n = 6$).

Although the influence of tannins on Fe absorption has been indicated as one of the main factors negatively affecting Fe availability by forming insoluble complexes, there are no known reports of their partition coefficients by using model membranes, as for flavonoids (Erlejman et al., 2004). A previous study demonstrated that high doses of hydrolysable tannins (15–240 μM) reduced the average lipid packing density of membranes in B14 cells (Chinese hamster) (Labieniec & Gabryelak, 2003). However, the latter authors indicated that the effect of tannins on membrane fluidity seems to be dependent on their chemical structure, and those compounds with high numbers of phenolic free hydroxyl groups would exhibit higher polarity, making their interaction within cell membranes difficult. In the present study, it is probable that low doses (1.3 and 6.6 μM, considering MW for tannic acid as 1700.12 g M^{-1}) of tannins are adsorbed to the polar head group of phospholipids in cell membranes, making the membrane richer in negatively charged groups and blocking Fe diffusion towards the brush border. This hypothesis would explain their marked negative effect on Fe absorption when pretreating the cell cultures.

3.4. Effect of green and black tea on Fe uptake

After the incubation period adequate cell viabilities were confirmed by MTT conversion percentages, ranging from 101–162%. The effects of green and black tea solutions on Fe ($FeSO_4$, $FeCl_3$, and FeEDTA solutions) uptake by Caco-2 cells are shown in Figs. 2, 3, and 4B. It is important to mention that both green and black tea solutions used in the present study had no effect on ferritin formation when cultures were exposed to solutions without Fe.

In $FeSO_4$ solutions, only the joint addition of total phenolics from green tea, in the proportion 1:1 (Fe:Grt), caused a significant ($p < 0.05$) decrease on Fe absorption relative to the independent addition of $FeSO_4$ solution. The same pattern could be observed with the joint addition of black tea. When cell cultures were not pre-treated, either with green or black tea solutions, there were no significant ($p > 0.05$) differences in ferritin values. In addition, increasing doses of total phenolics did not increase the inhibitory effect on Fe uptake, as concluded from the similar ferritin concentration values quantified. Overall, when cell cultures were pre-treated with black tea solutions, Fe uptake was decreased to a greater extent than with joint addition (within Fe-containing solutions) in *in vitro* media. This observation could be explained by the higher content of galloyl groups estimated in black tea solutions (Table 1).

The joint addition of total phenolics from green tea within Fe-containing ($FeCl_3$) solutions completely abolished the Fe uptake; ferritin concentrations in these cultures were similar ($p > 0.05$) to the controls. The black tea solutions inhibited Fe absorption as

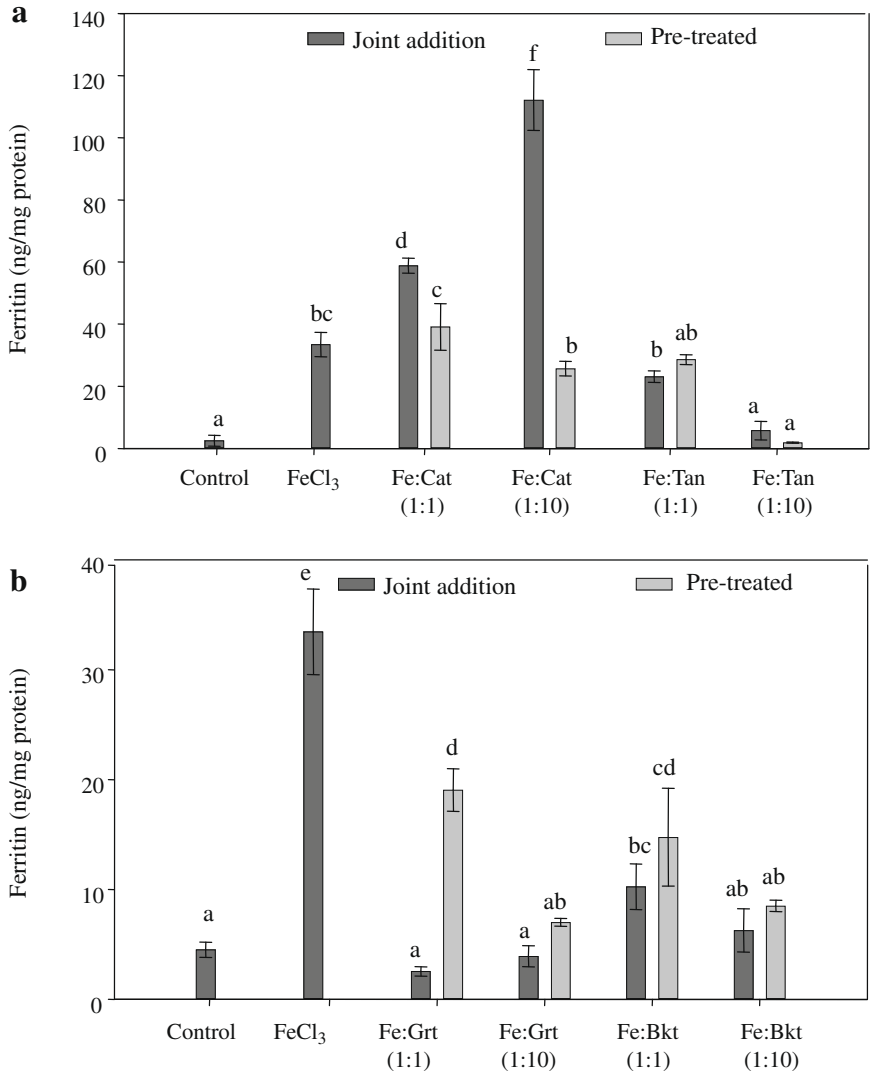

Fig. 3. Iron (Fe) uptake from $FeCl_3$ solution: (A) effect of catechin (Cat) or tannic acid (Tan), added during digestion process or pretreating of the cultures (2 h), on Fe uptake; (B) effect of green (Grt) and black (Bkt) tea, added during digestion process or pretreating of the cultures (2 h), on Fe uptake. Values are expressed as means ± standard deviation ($n = 6$).

well; however, when compared to the controls, the ferritin values were slightly increased ($p < 0.05$) when considering the lowest concentration assayed. Cell cultures pre-treated with both tea solutions and only at the lowest Fe/phenolics (1:1) proportion had increased ferritin values relative to the controls. Similar patterns to those described for $FeCl_3$ solutions were noted for FeEDTA solutions.

The addition of tea and/or its extracts to foods has been related to decreased non-heme Fe absorption in animals (Valdez et al., 1992) and humans (Samman et al., 2001) studies. Furthermore, a potential role of tea has also been suggested as a cause of Fe deficiency and subsequent microcytic anemia in infants (Merhav et al., 1985). The results obtained in this study are in good agreement with these previous reports. It has been accepted that phenolics reduce Fe availability by forming insoluble complexes with Fe facilitated largely by the galloyl groups (Brune et al., 1991). Accordingly, we observed a greater negative effect of tannic acid standard solutions on Fe absorption by Caco-2 cells (Figs. 2, 3 and 4A). However, ligand interactions need to be a consideration in real foods, and cannot be reflected by single phenolics-dosing experiments. This fact could explain the enhanced Fe uptake by catechin standard solutions. The positive effect of catechin, when added jointly within Fe-containing ($FeSO_4$ and $FeCl_3$) solutions, was not seen for green tea solutions, which had the highest catechin contents (Table 1), in either $FeSO_4$ or $FeCl_3$ solutions (Figs 2 and 3B). In addition, ferritin values were similar ($p > 0.05$) to those quantified in cultures exposed to black tea whose catechin content was 4.3-fold lower. It is important to mention that the presence of the 3',4',5'-trihydroxy group of epigallocatechin or epicatechin gallate has been associated with reduced Fe-binding ability (Khokhar & Apenten, 2003). The non-significant ($p > 0.05$) statistical differences in ferritin formation among both tea solutions might be explained by the high Fe-binding ability of the galloyl groups present. Thus, the lower content quantified in green tea solution (Table 1) would be enough to completely chelate the soluble Fe in *in vitro* media.

The increased ferritin values quantified in $FeCl_3$ and FeEDTA solutions can be explained, at least in part, by the fact that tea phenolics can interact with cell membranes (Erlejman et al., 2004; Verstraeten et al., 2003). Among the flavanols, the presence of a gallate residue at position #3 in epicatechin gallate and epigallocatechin gallate has been reported to determine a marked increase in the anti-disruption effect of membranes of liposomes, compared to epicatechin and epigallocatechin, by partitioning in the hydrophobic phase and subsequently increasing the membrane fluidity (Erlejman et al., 2004). In the present study, the 15-fold

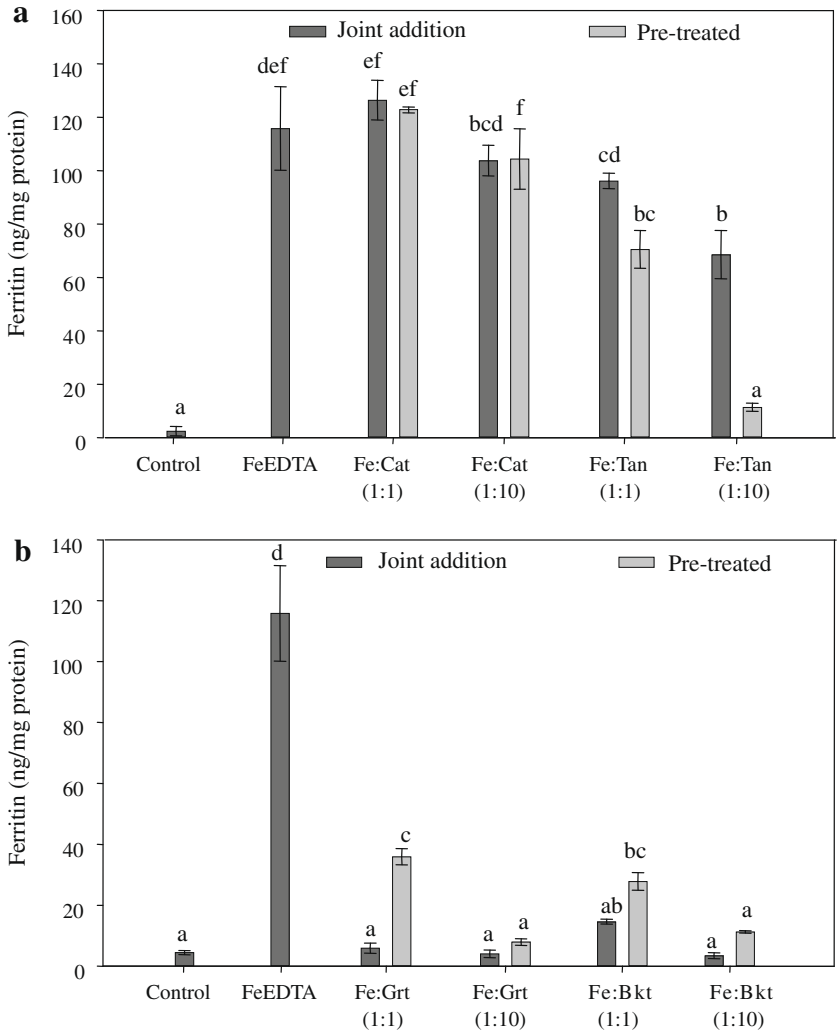

Fig. 4. Iron (Fe) uptake from FeEDTA solution: (A) effect of catechin (Cat) or tannic acid (Tan), added during digestion process or pretreating of the cultures (2 h), on Fe uptake; (B) effect of green (Grt) and black (Bkt) tea, added during digestion process or pretreating of the cultures (2 h), on Fe uptake. Values are expressed as means ± standard deviation ($n = 6$).

higher content of epigallocatechin-3-gallate in green than in black tea would help to explain the greater positive effect on Fe uptake produced by green tea relative to black tea in $FeCl_3$ and FeEDTA solutions.

The Fe uptake from $FeSO_4$ solutions, within black tea phenolics, in any treatments assayed, was only slightly ($p < 0.05$) reduced when compared with the marked negative effect, caused by tannic acid standard solutions (Fig. 1A and B), on Fe uptake from this same fortificant. Thus, an important consideration of Fe oxidation status is needed, though cell cultures pre-treated with either green or black tea had no marked effect on Fe uptake from $FeSO_4$ solutions.

In summary, these results suggest that Fe-chelating abilities of galloyl groups in tea solutions do not exhibit as much binding capacity as do tannic acid standard solutions. Furthermore, the results presented show that considerable caution is necessary when considering the effects on Fe uptake by single dosing experiments. When considering real tea solutions, which contains catechin and tannins, the effect on Fe absorption was markedly negative for their joint addition within Fe-containing solutions. Besides, upon pretreating cell cultures with tea solutions, before being challenged with Fe solutions, the ferritin values were slightly increased relative to the controls, although, in all assayed cases the Fe absorption was decreased compared to the exposure with Fe alone. It is noteworthy that lower inhibition was produced in $FeSO_4$ solutions; this result was not statistically ($p > 0.05$) significant when using the ratio 1:10 (Fe:tea) of both green and black tea solutions. The data presented contribute to improve the understanding of the mechanism(s) by which phenolics in tea may affect the diffusion of Fe towards the brush border membrane and the uptake processes in enterocytes (Caco-2 cells). However, further studies and complementary data within human trials are needed to confirm these mechanism(s). Improved understanding of the stated effects of phenolics on Fe availability and absorption are needed to help design adequate dietary strategies.

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