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Effect of Calcium, Tannic Acid, Phytic Acid and Pectin over Iron Uptake in an In Vitro Caco-2 Cell Model

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Abstract Calcium, phytic acid, polyphenols and fiber are major inhibitors of iron absorption and they could be found in excess in some diets, thereby altering or modifying the iron nutrition status. The purpose of this study is to evaluate the effect of calcium, tannic acid, phytic acid, and pectin over iron uptake, using an in vitro model of epithelial cells (Caco-2 cell line). Caco-2 cells were incubated with iron (10–30 μM) with or without $CaCl_2$ (500 and 1,000 μM) for 24 h. Then, cells were challenged with phytic acid (50–150 μM); pectin (50–150 nM) or tannic acid (100–500 μM) for another 24 h. Finally, ^{55}Fe (10 μM) uptake was determined. Iron dialyzability was studied using an in vitro digestion method. Iron uptake in cells pre-incubated with 20 and 30 μM Fe was inhibited by $CaCl_2$ (500 μM). Iron uptake decreased in cells cultured with tannic acid (300 μM) and $CaCl_2$ (500–1,000 μM) (two-way ANOVA, $p=0.002$). Phytic acid also decreased iron uptake mainly when cells were treated with $CaCl_2$ (1,000 μM) (two-way ANOVA; $p<0.05$). Pectin slightly decreased iron uptake (p=NS). Iron dialyzability decreased when iron was mixed with $CaCl_2$ and phytic or tannic acid (T test $p<0.0001$, for both) but not when mixed with pectin. Phytic acid combined with calcium is a strong iron uptake inhibitor. Pectin slightly decreased iron uptake with or without calcium. Tannic acid showed an unexpected behavior, inducing an increase on iron uptake, despite its low Fe dialyzability.

Keywords Iron uptake · Iron dialyzability · Calcium · Tannic acid · Pectin · Phytic acid

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Introduction

Iron is an essential micronutrient, being an important constituent of multiple proteins as an organic or inorganic cofactor [1]. Iron-containing proteins exert a variety of functions which include gas transport, electron transfer in the respiratory chain, bio-degeneration catalysts or biosynthesis, transcription factors, repressors, intermediate metabolism enzymes, and DNA synthesis and repair [1, 2].

Iron can be found as non-heme (inorganic) or heme iron. Iron absorption occurs mainly in the duodenum and upper jejunum. It has been suggested that heme iron enters the enterocyte using the putative heme transporter (HCP-1), and the uptake of non-heme iron is through the divalent metal transporter 1 (DMT-1) located in the apical membrane of enterocytes [3, 4]. Several factors influence the efficiency of intestinal absorption of inorganic iron but not heme iron. The solubility of non-heme iron is critical and many dietary compounds such as calcium, phytic acid, and polyphenols lead to the production of insoluble iron complexes [5]. Intestinal absorption of inorganic iron could be affected by the absence of dietary factors that stimulate its absorption, such as vitamin C and animal proteins [6]; also, iron absorption is influenced by body iron status and gender [6]. Also, iron uptake is severely affected in pathologic conditions such as inflammatory bowel disease [7].

Calcium is a well-documented inhibitor of iron absorption. Initially, it was thought to exert its effect in the lumen of the gastrointestinal tract. However, recent studies suggest that this inhibition may occur at both the apical and basolateral membranes of enterocytes [8]. Potential mechanisms of calcium activity include alterations in the balance of intraluminal ligands, changes in gastrointestinal transit time, decreased iron uptake by transporter competition, and Fe transport interference in mucosal cells [9]. There are other compounds besides calcium that inhibit non-heme iron uptake, such as phytic acid

and polyphenols. Phytic acid is a component of cereals and is recognized as one of the major inhibitors of iron absorption [10]. Despite this fact, iron fortification of flour and other cereals is the main measure adopted in countries where anemia is highly prevalent [11], and it is documented that even small amounts of phytic acid in food will significantly reduce iron absorption [12]. The negative effect of phytic acid on iron absorption has been shown to be dose dependent and starts at very low concentrations of 2–10 mg phytic acid/meal [11]. The molar ratio of phytic acid and iron should be <1:1 or preferably <0.4:1 to improve iron absorption in meals that do not contain any iron absorption enhancers [13].

Bioactive dietary polyphenols have increasingly attracted attention due to their reported health benefits and because they also possess the capability to chelate metals [14]. Tea, red wine, and other beverages are rich in polyphenolic compounds. A high intake of dietary polyphenolic compounds may have important consequences on iron status. On the other hand, the effect of dietary fiber on iron bioavailability is also the focus of many studies, since fiber intake has been stimulated in multiple nutritional guidelines as a way to reduce the energy value of diets.

Pectin is a cell wall polysaccharide of all higher plants and is an important water-soluble component of dietary fiber. Pectin is a polymer of D-galacturonic acid residues that are usually esterified to various degrees with methanol [15]. In solutions, the carboxyl groups of unesterified units may form complexes with poly-valent metals such as calcium, magnesium, and iron [15]. However, the in vivo and in vitro effect of pectin on iron uptake is controversial [15–17]. Such disagreements could have been due to differences in pectin concentrations, but also in the binding degree of pectin to iron. Pectin binding to minerals and cholesterol varies depending on its molecular weight, esterification degree, and distribution mode of its free carboxylic groups [18].

In this study, our aim was to investigate the effect of calcium, tannic acid, phytic acid, and pectin over iron uptake and dialyzability in order to determine iron solubility under these conditions, using an in vitro model with Caco-2 cells.

Methods

Cell Culture Caco-2 cells were kept at 37 °C in DMEM medium supplemented with 10 % FBS, without an essential amino acid and antibiotic–antimicotic mix, at 95 % O_2 and 5 % CO_2. The medium was changed every 2 days. Cells were seeded in a six-plate well for 5 days until they reached a 75 % confluence. Then, cells were challenged with phytic acid (50–150 μM) (rice, IP6, sodium salt; Sigma Aldrich), pectin (50–150 nM) (esterified potassium salt from citrus fruit; Sigma Aldrich) or tannic acid (100–500 μM), and 10 μM of Fe-NTA (1:2.2 $FeCl_3$-Nitriloacetate) in the presence or absence of $CaCl_2$ (500 and 1,000 μM) for 24 h. Control group cells were incubated with DMEM and 10 μM of Fe-NTA.

^{55}Fe Uptake Iron transfer from the apical compartment to the cell was determined. After a 5-day incubation, cells were incubated at 37 °C for 1 h with 10 μM ^{55}Fe in transport buffer (50 mM HEPES, 130 nM NaCl, 1 mM $MgSO_4$, 10 mM KCl). Finally, cells were washed three times with cold PBS, removed with Tris-EDTA and centrifuged for 2 min at 10,000×*g*. Pellets were resuspended in a $NaHCO_3$ 2 %/NaOH 0.1 N solution. ^{55}Fe was quantified in glass vials by liquid scintillation counting. Cellular protein concentrations were assessed using the Lowry method [19]. In order to determine the iron concentrations used in all experiments, cells were challenged with increasing doses of iron (10, 20, and 30 μM) and two calcium concentrations (500 and 1,000 μM). Fe uptake was determined at 10 μM, remaining unaffected at any of both $CaCl_2$ concentrations.

In Vitro Digestion and Iron Dialyzability After homogenate preparation, the following mixtures were analyzed: (1) 300 μM Tannic acid, 10 μM Fe-NTA, with or without 1,000 μM $CaCl_2$; (2) 100 nM Pectin, 10 μM Fe-NTA, with or without 1,000 μM $CaCl_2$; (3) 100 μM Phytic acid, 10 μM Fe-NTA, with or without 1,000 μM $CaCl_2$. To determine iron solubility in the extracts, 2 mL of pepsin (0.2 g pepsin/5 mL 0.1 mol/L HCl; Sigma Chemical, Saint Louis, MO) were added to the homogenate (pH 2.0) and incubated for 2 h at 37 °C in a shaker at 5.000 rpm. To determine Fe-NTA dialyzability, a dialysis bag with 20 mL Pipes buffer (0.1 M, pH 7.3) was introduced in the homogenate and incubated for 30 min in a shaker at 5.000 rpm. Afterwards, pH was raised to 6 with 1 M $NaHCO_3$, and 5 mL of bile/pancreatin solution (0.05 g pancreatin; 0.3 g bile extract, glycine, taurine conjugates, and other bile salts; Sigma Aldrich) were added to 25 mL of 0.1 N $NaHCO_3$, and incubated for 90 min at 37 °C. After incubation, both the homogenate and Pipes buffer were weighed. Total iron content was determined by atomic absorption spectroscopy with graphite furnace (Simaa 6100, Perkin Elmer) in (a) Pipes solution and (b) initial homogenate. Fe dialyzability was determined using the following formula:

$$\text{Fe dialyzability } (\%) = \frac{\left[\text{Pipes Fe}\left(\mu g/g\right) \times \text{final Pipes (g)}\right] + \left[\text{Pipes Fe}\left(\mu g/g\right) \times \text{final homogenate (g)}\right]}{\text{Initial Fe homogenate}\left(\mu g/g\right) \times \text{initial homogenate (g)} \times 100}$$

Where: (a) Pipes Fe (in microgram per gram)=iron concentration in Pipes buffer after dialysis; (b) final Pipes (in gram)= Pipes buffer weighed after dialysis; (c) final homogenate (in gram): homogenate weighed after dialysis; (d) initial homogenate Fe (in microgram per gram)=homogenate iron concentration before dialysis and (e) initial homogenate (in gram)= homogenate weighed before dialysis.

Statistical Analysis

Results are expressed as means±SEM. For cellular experiments, results are presented as the ratio between ^{55}Fe uptake of cells treated with or without $CaCl_2$ and challenged with tannic acid, phytic acid or pectin, and Fe-NTA; and cells treated only with Fe-NTA. A two-way ANOVA test was applied for treatment analysis. *T* tests between treatments with or without $CaCl_2$ were used for iron dialyzability. All statistical analyses were performed with GraphPad Prism 4 software. Statistical significance was assigned to $p<0.05$.

Results

^{55}Fe uptake in cells incubated with 20 and 30 μM Fe, was only inhibited by 500 μM of calcium (Fig. 1). In cells pre-incubated with a 10 μM concentration, ^{55}Fe uptake was not affected at any calcium concentration (two-way ANOVA: calcium treatment $p<0.014$; iron treatment $p<0.018$ and interaction p=NS; Fig. 1).

Iron uptake in cells incubated with different tannic acid concentrations (300–500 μM) increased at all the analyzed concentrations compared to the control group (without tannic acid and calcium) (Fig. 2). The two-way ANOVA analysis indicated that $CaCl_2$ treatment was significant ($p<0.001$); however, tannic acid treatment ($p<0.001$) showed no interaction between treatments (p=NS). After calcium addition, the stimulatory effect was mainly reversed in cells incubated with tannic acid (400 μM)/calcium (500 μM).

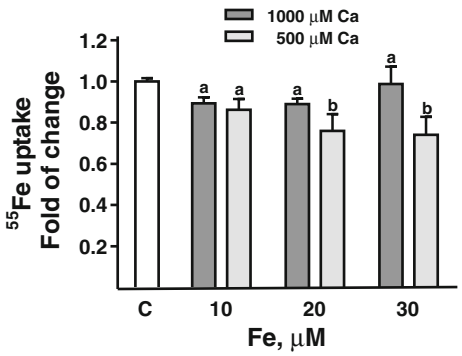

Fig. 1 ^{55}Fe uptake in Caco-2 Cells cultured with increasing Fe-NTA concentrations and with 1,000 and 500 μM of $CaCl_2$. Results are means± SEM and are the ratio between ^{55}Fe uptake of the treated and control cells (cells without $CaCl_2$). C: Control group, cells incubated with DMEM/10 μM of Fe-NTA. Bonferroni post-test: *different letters* within groups mean $p<0.05$

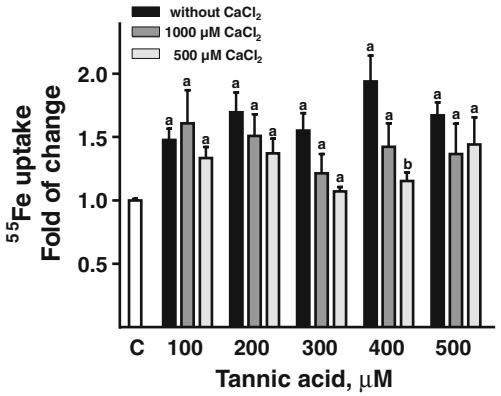

Fig. 2 ^{55}Fe uptake in Caco-2 cells challenged with Fe-NTA 10 μM, Tannic acid (100, 200, 300, 400, 500 μM), with or without $CaCl_2$ (1,000, 500 μM). Results are means±SEM and are the ratio between ^{55}Fe uptake of treated and control cells. C: Control group, cells incubated with DMEM/10 μM of Fe-NTA. Bonferroni's post-test: *different letters* within groups mean $p<0.05$

An inhibitory effect over iron uptake was observed at different pectin concentrations (50, 100 or 150 nM) (Two-way ANOVA: pectin treatment ($p<0.001$)), as well as during $CaCl_2$ treatment and interaction (p=NS) (Fig. 3).

Phytic acid (50, 100 or 150 μM) alone or combined with calcium induced iron uptake inhibition (two-way ANOVA: $CaCl_2$ treatment $p<0.001$; phytic acid treatment $p<0.001$ and interaction $p<0.001$) (Fig. 4). We observed that inhibition was increased in the presence of 1,000 μM calcium. Also, 500 μM $CaCl_2$ induced a decreased iron uptake when cells were cultured with 150 μM of phytic acid.

Furthermore, iron dialyzability decreased when iron was incubated with tannic acid or phytic acid and $CaCl_2$ (Table 1). We observed that the percentage of iron dialyzability with pectin was low in the presence of $CaCl_2$. Iron dialyzability

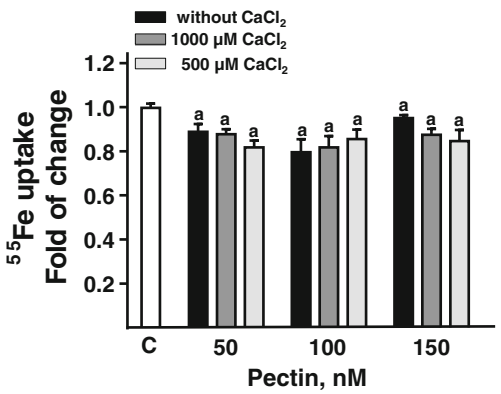

Fig. 3 ^{55}Fe uptake in Caco-2 cells challenged with Fe-NTA 10 μM, Pectin (50, 100, 150 nM), with or without $CaCl_2$ (1,000, 500 μM). Results are means±SEM and the ratio between ^{55}Fe uptake of the treated cells and control cells. *C*: Control group, cells incubated with DMEM/10 μM of Fe-NTA. Bonferroni's post-test: *different letters* within groups mean $p<0.05$

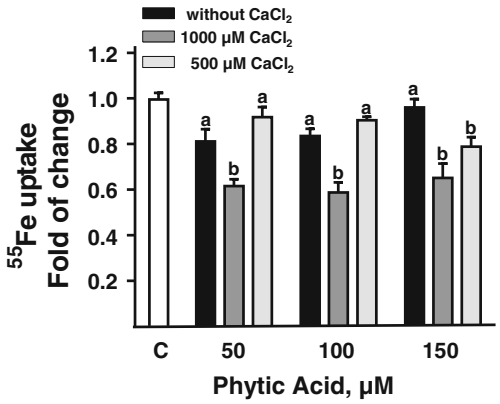

Fig. 4 ^{55}Fe uptake in Caco-2 cells challenged with Fe-NTA 10 μM, phytic acid (50, 100, 150 μM), with or without $CaCl_2$ (1,000, 500 μM). Results are means±SEM and are the ratio between ^{55}Fe uptake of treated and control cells. *C*: Control group, cells incubated with DMEM/10 μM of Fe-NTA. Bonferroni's post-test: *different letters* within groups mean $p<0.05$

with pectin was not different in the presence of $CaCl_2$; however, the percentage of dialyzability was low (Table 1).

Discussion

Throughout this study, we investigated the relationship between calcium and recognized iron absorption inhibitors such as tannic acid, phytic acid, and fiber (as pectin) in an in vitro model. We found that calcium (1,000 and 500 μM $CaCl_2$) inhibits iron uptake in cells pre-incubated with Fe (10 to 30 μM) approximately from 10 to 20 %. However, this inhibition was significant when cells were incubated with 500 μM $CaCl_2$ as well as 20 and 30 μM iron. The mechanism by which calcium affects iron absorption remains unclear and open to debate. Also, Thompson et al. [9] found that calcium (1.25 mM $CaCl_2$) reduced iron bioavailability by decreasing DMT1 expression at the apical cell membrane in an in vitro study, thereby down-regulating iron transport into the cell. In our study, we found that 1,000 μM $CaCl_2$ reduces iron uptake approximately in a 20 % at all iron concentrations. This was mainly observed in cells incubated with 500 μM of $CaCl_2$ and higher iron concentrations.

Table 1 Dialyzability of iron combined with tannic acid (300 μM), pectin (100 nM), phytic acid (100 μM), and with or without $CaCl_2$ (1,000 μM)

Compounds	Dialyzability (%)		*p* value[a]
	Without $CaCl_2$	With $CaCl_2$	
Tannic acid	37.1 (20.6–66.7)	17.6 (9.1–34.4)	<0.0001
Phytic acid	32.5 (15.9–66.5)	19.7 (16.8–23.2)	<0.0001
Pectin	42.7 (23.6–77.1)	29.8 (24.4–36.5)	>0.05

Results are expressed as geometric means and range (±1DE)

[a] Analysis by *T* test

There are other components in the diet such as fiber, phytic acid, and polyphenols that reduce iron absorption [5]. Dietary fiber encompasses various complex substances, mainly non-starch polysaccharides and lignin [20]. These non-digestible carbohydrates can be insoluble, like cellulose and certain hemicellulose, or soluble, generally pectins, β-glucans, gums, mucilages, and oligosaccharides. Non-digestible polysaccharides have important health benefits, among which are to promote normal laxation, reduce the risk of some cancers [21] and cardiovascular diseases, as well as adult onset of diabetes mellitus [22]. However, there are concerns that high-fiber diets also have adverse effects such as the decrease in the bioavailability of essential mineral and trace elements. In Caco-2 cells challenged with 50, 100, and 150 nM pectin and $CaCl_2$, we observed that pectin reduced iron uptake in approximately 15 %, independent of calcium concentrations (1,000 or 500 μM). We also showed that iron dialyzability decreased in the presence of pectin, without differences during calcium treatments. In previous studies, Bosscher et al. [20] reported that soluble fiber reduced iron and zinc availability using a formula thickened with gum and guar gum. Also, pectin has a high content of carboxyl groups, with a high binding ability to cations at a neutral pH. In these conditions, pectin electrostatically interacts with mineral cations [23]. In the small intestine (pH 6.5–7.0), carboxyl groups are deprotonated; and in solution, the carboxyl groups of the unesterified units of pectin can bind cations such as Ca, Mg, and Fe (assuming that pectin would reduce mineral bioavailabilty) [23]. However, if the binding of mineral cations by pectin depends on carboxyl groups, this binding could be reversible and would then be affected by pH, ionic strength, and temperature [23]. There are few studies that have reported iron bioavailability when bound to pectin. Miyada et al. [24] found that rats fed with pectin showed increased hemoglobin concentrations, suggesting that iron bound to pectin is used by rats. In our study, pectin was 75 % esterified and the pH of the medium was near 7.0. This would indicate the chance that carboxyl groups bind iron; yet, we can only conclude in regards to the relationship between pectin and iron uptake, but not on iron bioavailability since fermented pectin may release bound iron in the large intestine.

On the other hand, we studied the action of phytic and tannic acid over iron uptake. We found that tannic acid did not inhibit iron uptake in Caco-2 cells; in fact, polyphenol increased iron uptake approximately from 15 to 20 % at 100 and 200 μM with and without $CaCl_2$. Iron uptake only decreased in cells treated with 300 μM of tannic acid and $CaCl_2$ (1,000 and 500 μM) compared to the control, but not to the control without calcium and tannic acid; hence, uptake reduction was calcium dependent.

Tannic acid is a polyphenol that is found in tea, wine, coffee, and other beverages [25]. Few studies have shown that tannic acid could decrease iron uptake and its bioavailability [26, 27]. In our cellular model, we could not demonstrate a reduction in iron availability; nevertheless, the fact that tannic acid can increase iron absorption at high concentrations may be related to an increase in cell viability, as high tannic acid concentrations increased cell viability by 40 % (data not shown).

Phytic acid is one of the most recognized inhibitors of iron bioavailability. Phytate can decrease bioavailability of critical nutrients such zinc, iron, calcium [28]. Phytic acid exerts its inhibitory effect on mineral absorption by forming insoluble and indigestible complexes [29]. We found that phytic acid (50–150 μM) in the presence of 500 and 1,000 μM calcium, inhibited iron uptake between 20 to 40 %, respectively. Without calcium, iron uptake was decreased only from 4 to 20 %. The decrease of iron uptake can, in part, be explained by the dialyzability of iron when combined with phytic acid and calcium. We found that iron dialyzability was significantly decreased in this condition compared to the homogenate without $CaCl_2$. The interaction between phytic acid and iron is especially important; iron deficiency prevention has been approached through fortification of flour and cereals, both main sources of phytic acid. In our study, we were not able to demonstrate that phytic acid and calcium have a synergic or additive effect over iron uptake, despite having showed that iron dialyzability, in the presence of calcium, was significantly decreased.

In this study, we demonstrated that iron uptake was reduced with calcium and phytic acid. This decrease was related to decreased iron dialyzability. With pectin, iron uptake and dialyzability was also reduced, but this decrease was not related to calcium. In this model, we could not demonstrate that tannic acid induced a reduction in iron uptake; nevertheless, we observed that iron dialyzability was reduced. This result could be explained by the increased cellular viability observed in Caco-2 cells exposed to increased concentrations of tannic acid. Finally, the results suggest that it is necessary to be cautious with the interaction between iron, calcium, and phytic acid in meals.

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