The Effect of Calcium, Ascorbic Acid and Tannic Acid on Iron Availability from *Arthrospira Platensis* by Caco-2 Cell Model

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ABSTRACT

There are several dietary factors that are known to affect the bioavailability of iron such as protein, calcium, ascorbic acid, polyphenol and phytate. The aim of this study was to determine iron bioavailability from spirulina (*Arthrospira platensis*) in the presence of calcium, ascorbic acid (AA) and tannic acid (TA). The experiments were conducted using *in vitro* digestion/Caco-2 cell culture system. Comparison was made with ferrous sulphate ($FeSO_4$) as a reference. At low calcium concentrations (1:05, 1:10, 1:15, 1:20 Fe:Ca molar ratios), iron bioavailability from spirulina was significantly better than from $FeSO_4$. However, at higher calcium concentrations (1:37, 1:75, 1:149 Fe:Ca molar ratios), iron bioavailability from spirulina was found to be similar to that from $FeSO_4$. Addition of ascorbic acid of different concentrations (1:0.5, 1:1, 1:1.5 and 1:2 Fe:AA molar ratios) increased iron availability from $FeSO_4$ more than from spirulina. The concentrations of tannic acid (1:1, 1:1.4 and 1:2 Fe:TA molar ratios) used in this study reduced iron availability from spirulina but not from $FeSO_4$. Iron from spirulina by itself is highly bioavailable but consumers should be aware of factors that could inhibit its bioavailability.

INTRODUCTION

Arthrospira platensis or commonly known as spirulina, is a microscopic and filamentous cyanobacterium (blue-green algae) that has a long history of use as food. Spirulina is available commercially as a health food for human containing nutrients including protein (65% of dry weight) and various vitamins and minerals, especially iron (Dillon, Phuc & Dubacq, 1995; Kay, 1991). Various studies on iron bioavailability from spirulina have shown that it is not only a good source of iron but more importantly the iron is highly bioavailable (Puyfoulhoux *et al.*, 2001; Kapoor & Mehta, 1993). However, dietary factors that may influence the bioavailability of iron from spirulina have not been studied so far.

The objective of this study was to determine the effect of three dietary factors namely calcium, ascorbic acid and tannic acid on iron availability from spirulina in comparison with ferrous sulphate ($FeSO_4$), using an in vitro diges-

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tion/Caco-2 cell culture method. The potential of using Caco-2 cells as a model for studying iron bioavailability has been highlighted in various studies (Glahn *et al.*, 1998; Serfass & Reddy, 2003). Caco-2, when grown on microporous membranes in bicameral chambers, differentiates spontaneously into bipolar enterocyte that exhibit many of the characteristics of normal epithelial cells. These include microvilli of the brush border membrane, tight intercellular junctions, the excretion of brush border associated enzymes and dome formation (Vachon & Beaulieu, 1992). The design of the bicameral chambers permits study of iron uptake from the apical chamber, transport into the cell and vectorial secretion of iron into the basal chamber.

MATERIALS AND METHODS

All the enzymes and chemicals were obtained from Sigma Chemical (St. Louis, MO, USA) unless noted otherwise. Deionized water was used throughout the study unless noted otherwise.

Spirulina culture

The strain *Arthrospira platensis* (Nordstedt) Gomont (ATCC 29408) was grown in a 150 liter tank under 12 h light-dark cycle in modified kosariks medium at pH 8 and temperature 27°C. This medium contained $NaHCO_3$ 9.00 g/L, K_2HPO_4 0.25 g/L, $NaNO_3$ 1.25 g/L, K_2SO_4 0.50 g/L, NaCl 0.50 g/L, $MgSO_4.7H_2O$ 0.70 g/L, $CaCl_2$ 0.20 g/L, $FeSO_4.7H_2O$ 0.005 g/L and A_5-micronutrients 0.5 ml/L and was supplied with a light aeration (30 l/min). The A_5-micronutrients consist of H_3BO4_4 2.86 g/L, $MnCl_2.4H_2O$ 1.81g/L, $ZnSO_4.7H_2O$ 0.22 g/L, $CuSO_4.5H_2O$ 0.08 g/L, MoO_3 0.01 g/L, and $CoCl_2.6H_2O$ 0.01 g/L. After 2 weeks, the biomass was recovered through centrifugation at 6, 000 rpm for 10 mins. It was then thoroughly washed, frozen and freeze-dried.

Cell culture

Caco-2 cells, originating from human colorectal carcinoma, were purchased from the American Type Culture Collection (ATCC, Rockville, USA) at passage 18, and used in experiments at passages 25-31. Caco-2 cells were routinely maintained and expanded in Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island, NY, USA) with 10 % (v/v) fetal calf serum (PAA, Linz, Austria), 1 % (v/v) non-essential amino acids and 1 % (v/v) antibiotic solution (Gibco BRL) and were grown at 37°C in an incubator with a 5 % carbon dioxide (CO_2)/95 % atmosphere air at constant humidity. When the cells were 80-90% confluent, they were rinsed with EDTA solution before being dissociated using Tyrpsin-EDTA and re-suspended in a 75 cm^2 culture flask (Corning Costar, Cambridge, MA, USA) for continued growth.

For iron uptake experiments, cells were seeded at a density of 5 x 10^4 cells/cm^2 in Transwell bicameral chambers with a pore size of 3.0 µm (Corning Costar) coated with collagen. The collagen film was applied to the filter as 50 ml of collagen solution (0.1 mg/ml; rat tail type I). Cells were used for iron uptake experiments at 13 day postseeding. The development of functional tight junctions and the integrity of the monolayer during differentiation of Caco-2 cells were monitored by determining transeptihelial electrical resistance (TEER) and diffusion of phenol red across the monolayers. The TEER was measured on alternate days after seeding using the Millicell-ERS apparatus (Millicell-ERS Voltohmmeter, Millipore, Japan). The diffusion of phenol red was determined by measuring the optical density (550 nm) of both the apical and basal chamber using a universal microplate reader (Bio-Tek Instrument, Inc., ELX800, USA). Cell layers were used only after

TEER had increased to greater than 250 $\Omega.cm^2$ and phenol red diffusion was less than 10%, both indicating the formation of a polarised intact monolayer.

In vitro digestion

All the reagents were prepared fresh before each experiment. $^{59}FeSO_4$ in 0.05 mol/L H_2SO_4 (NEN Life Science, Boston, USA) was mixed with the sample to achieve 0.148 MBq of ^{59}Fe. The amount of spirulina and $FeSO_4.7H_2O$ (BDH, Poole, England) used was 0.1244 g and 0.278 mg respectively to achieve a total iron concentration of 100 µmol/L in the initial 10 mL sample. The $^{59}FeSO_4$ was added to the sample between 12 to 16 hours before the experiment and kept at 4^OC (Glahn *et al* ., 1998; Gangloff, *et al* ., 1996). This method was based in part on studies by Garcia, Flowers & Cook (1996) and Au & Reddy (2000). The pH of the mixture was brought to 2.0 with 5.0 mol/L HCl before adding 0.5 mL pepsin per 10 mL sample and then incubated for 1 h at 37^OC in a shaking water bath. Following incubation, pH was raised to 6.7 with slow addition of 1 mol/L $NaHCO_3$ dropwise, and 2.5 mL pancreatin-bile per 10 mL original sample. Incubation was continued for 2 h more at 37^OC to mimic duodenal digestion. The digests were subjected to centrifugation at 15,000 x g for 5 min. Volume of supernatant was measured and the supernatant was used for iron uptake study.

Iron uptake by Caco-2 cells

At 13 d postseeding, cells were used for iron uptake experiments. Prior to the beginning of the uptake measurements, cell monolayers were rinsed twice with 1 mL of (Hanks' Balanced Salt Solution) HBSS. Then complete DMEM was added to the lower chamber. To begin the iron uptake, 1.5 mL of radiolabeled dialysate was layered over the cells in each well. The plates were incubated for 1 hour at 37^OC,

and uptake was terminated by aspirating the dialysates and immediately removed with 4^OC "stop" solution (140 mM NaCl and 10 mM PIPES; 3 volumes of 1 ml). To remove surface iron that was not tightly associated with the monolayer, cells were incubated for 10 min in a "removal" solution (140 mM NaCl and 10 mM PIPES, pH 6.7, containing 5 mM bathophenanthrolinedisulfonic acid and 5 mM sodium dithionite at 4^OC) (Glahn *et al* ., 1995). After 10 min the removal solution was aspirated, and the monolayers were rinsed three more times with stop solution. The monolayers were then solubilized in 1 mL 0.5 M NaOH. The contents of the apical, basal chambers and cells were counted using the Packard Cobra 5005 automatic gamma counter (Packard, USA).

Experimental Design

Experiments involving cell cultures were conducted in triplicate on the same day. The first series of experiments were to determine the effects of calcium on the bioavailability of iron from spirulina. Fifty six microgram of iron from $FeSO_4$ and spirulina were combined with varying amounts of calcium carbonate to yield Fe:Ca molar ratios of 1:05, 1:10, 1:15, 1:20 1:37, 1:75 and 1:149. Each of the solutions was then subjected to *in vitro* digestion.

The rest of the series of experiments were conducted in an identical fashion as the above, except ascorbic acid (AA) or tannic acid (TA) was used in place of calcium carbonate. For experiments using AA, Fe:AA molar ratios of 1:0.5, 1:1, 1:1.5 and 1:2 were used. As for TA, Fe:TA molar ratios of 1:1, 1:1.4 and 1:2 were used. All ratios used were adapted from various studies that use these ratios (Glahn *et al* ., 2000; Garcia-Casal, Leets & Layrisse, 2000).

Statistical analysis

Data are given as mean ± SEM and

they were analysed by one way analysis of variance (ANOVA) with Duncan's multiple range test for comparing among groups, using the Statistical Package for Social Sciences (SPSS) version 10.0. A significance level of $P < 0.05$ was adopted for all comparison.

RESULTS

Effect of Calcium

Addition of calcium carbonate ($CaCO_3$) significantly reduced solubility of iron from ferrous sulphate ($FeSO_4$) by about 80% for all the molar ratios (1:05, 1:10, 1:15, 1:20, 1:37, 1:75, 1:149 Fe:Ca molar ratios) (Figure 1). In spirulina, the inhibition is about 53% for molar ratios of

1:05, 1:10, 1:15, 1:20 and about 66% for 1:37, 1:75 and 1:149 Fe:Ca molar ratios. In the $FeSO_4$ group, there was no significant difference among the different molar ratios used in their ability to reduce iron solubility. However, in the case of spirulina, higher molar ratios (1:37, 1:75, 1:149 Fe:Ca molar ratios) significantly reduced iron solubility more than the lower molar ratios (1:05, 1:10, 1:15, 1:20 Fe:Ca molar ratios).

The amount of iron taken up by Caco-2 cells differed significantly between the spirulina and $FeSO_4$ groups for all Fe:Ca molar ratios used (Figure 2). Iron uptake at the lower Fe:Ca molar ratios (1:05 to 1:20) did not show significant differences within the $FeSO_4$ and spirulina groups. Iron uptake was significant when the Fe:Ca molar ratio was higher than 1:37 for spirulina but not for $FeSO_4$.
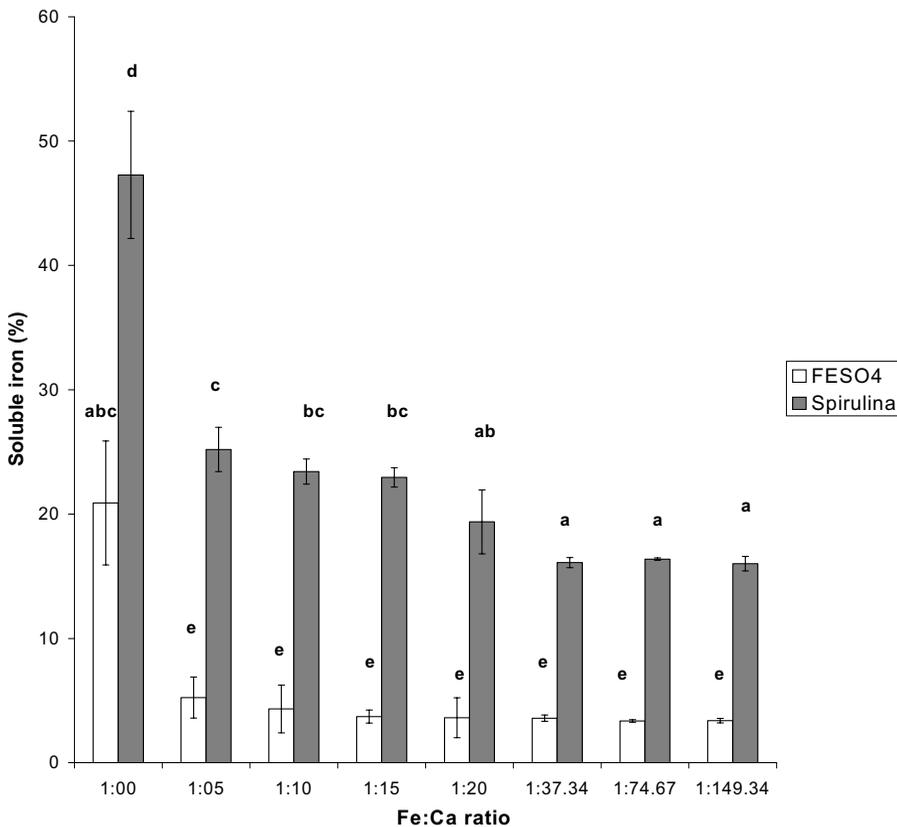

Figure 1. Percentage of soluble iron present after centrifugation for different iron to calcium molar ratios. Values are mean ± SEM, n=3-10. Bar values with no letters in common are significantly different (P<0.05)
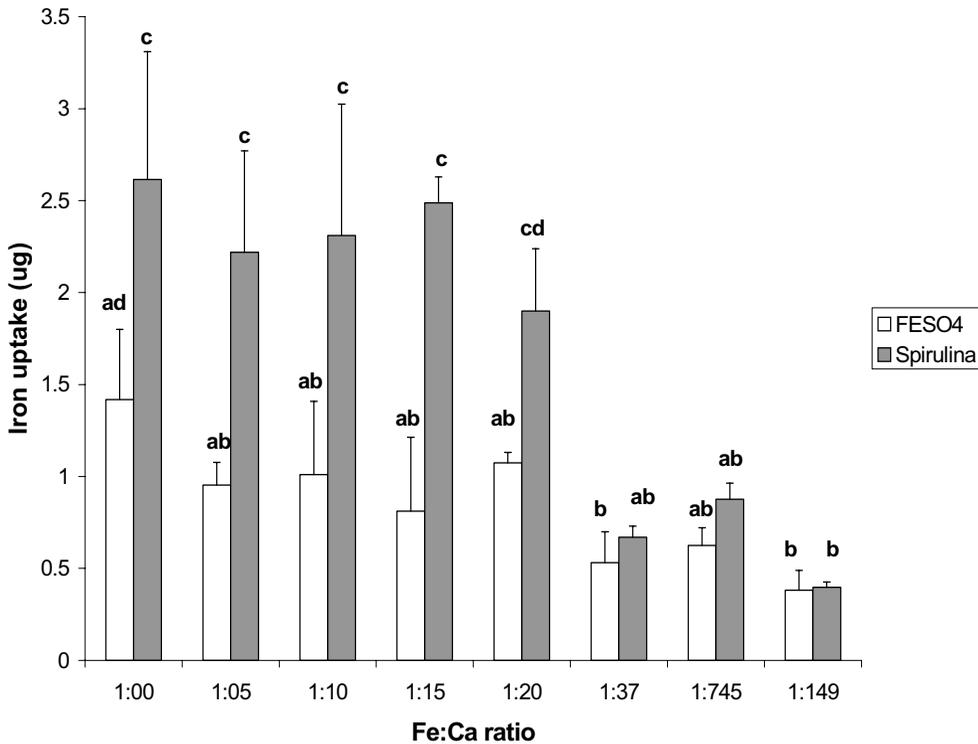
Figure 2. Total iron uptake by Caco-2 cell at different iron to calcium molar ratios. Total iron uptake was calculated by multiplying the % radioiron uptake by iron content in the medium. Values are mean $\pm$ SEM, n=3-10. Bar values with no letters in common are significantly different (P<0.05)

Effect of Ascorbic acid

The addition of ascorbic acid of different molar ratios (1:0.5, 1:1, 1:1.5 and 1:2 Fe:AA molar ratios) increased iron solubility up to about 300% in $FeSO_4$ in a dose dependant manner (Figure 3). In spirulina, ascorbic acid also increased iron solubility significantly (35%) but only at higher molar ratios (1:1.5 and 1:2 Fe:AA molar ratios). The Fe:AA molar ratios of 1:0.5 and above significantly increased iron uptake in $FeSO_4$ and spirulina (Figure 4). Figure 4 showed that on the average ascorbic acid

enhanced iron uptake 4 times more in $FeSO_4$ compared to spirulina, which was only enhanced 2 times more in the presence of ascorbic acid. Even though ascorbic acid enhanced iron solubility from spirulina less than $FeSO_4$, the iron uptake from spirulina was greatly increased compared to $FeSO_4$.

Effect of Tannic acid

All the three molar ratios (1:1, 1:1.4 and 1:2 Fe:TA molar ratios) reduced iron solubility in $FeSO_4$ and spirulina (Figure
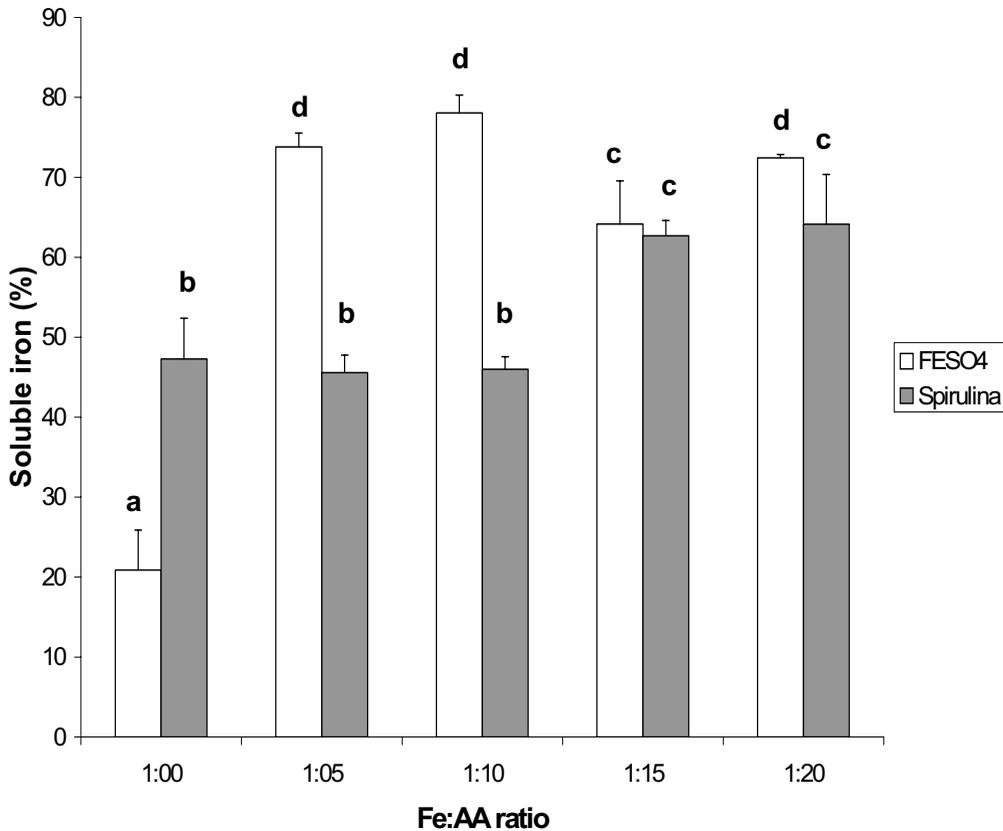
Figure 3. Percentage of soluble iron present after centrifugation at different iron to ascorbic acid (AA) molar ratios. Values are mean $\pm$ SEM, n=3-10. Bar values with no letters in common are significantly different (P<0.05)

5). The addition of tannic acid in *spirulina* group reduced iron solubility more than the FeSO$_4$ group. Actual amount of iron uptake was not significantly affected by all the different molar ratios of tannic acid in the FeSO$_4$ group (Figure 6). As for the spirulina group, the addition of the 3 different molar ratios of tannic acid significantly lowered the amount of iron uptake compared to spirulina alone.

DISCUSSION

The strength of the bond with which the ligand complexes iron may contribute to either enhancement or inhibition of iron absorption, and the stability of the iron-chelate increases with the concentration of the chelating ligand. Enhancers of iron absorption bind iron securely to maintain the stability of the bond and the solubility of the complex through the gastrointestinal tract. The strength of the iron-chelate bond, the solubility of the complex, and
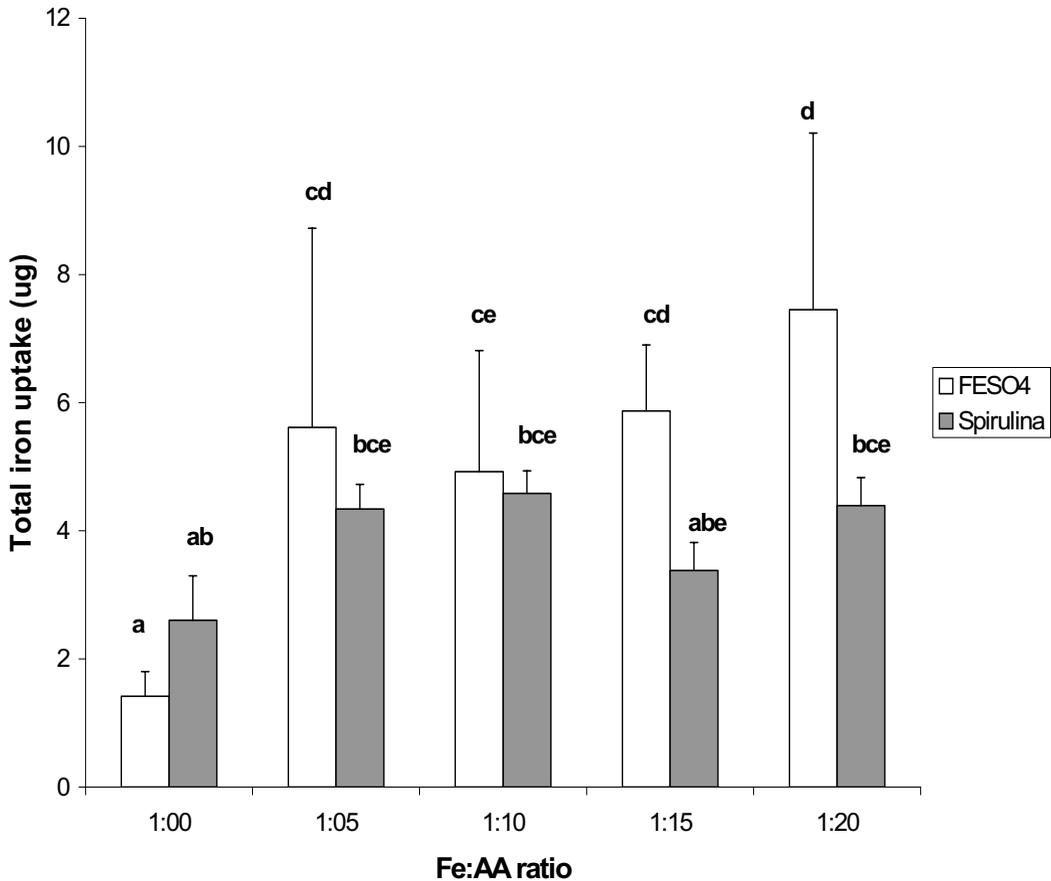
Figure 4. Total iron uptake by Caco-2 cell at different iron to ascorbic acid (AA) molar ratios. Total iron uptake was calculated by multiplying the % radioiron uptake by iron content in the medium. Values are mean $\pm$ SEM, n=3-10. Bar values with no letters in common are significantly different (P<0.05)

environmental factors such as pH and the presence of other competing chelators, determine whether iron is available for uptake by mucosal cells (Van Dokkum, 1992). Iron bioavailability is, therefore, determined by the degree of the affinity of each ligand for iron and the solubility of the iron-ligand complex.

Various studies have documented that calcium inhibited iron absorption (Reddy & Cook,. 1997; Minihane & Fairweather-Tait, 1998). Although some of

these studies showed conflicting results and different degrees of inhibition, the evidence clearly indicates that calcium present at levels common in meals and from ingestion of supplements inhibits the absorption of both haem and nonhaem iron (Hallberg, 1998).

In the present study, the molar ratios of calcium to iron are within a range that has been shown to inhibit iron absorption in humans (Hallberg *et al*., 1991). The degree of reduced solubility of iron by
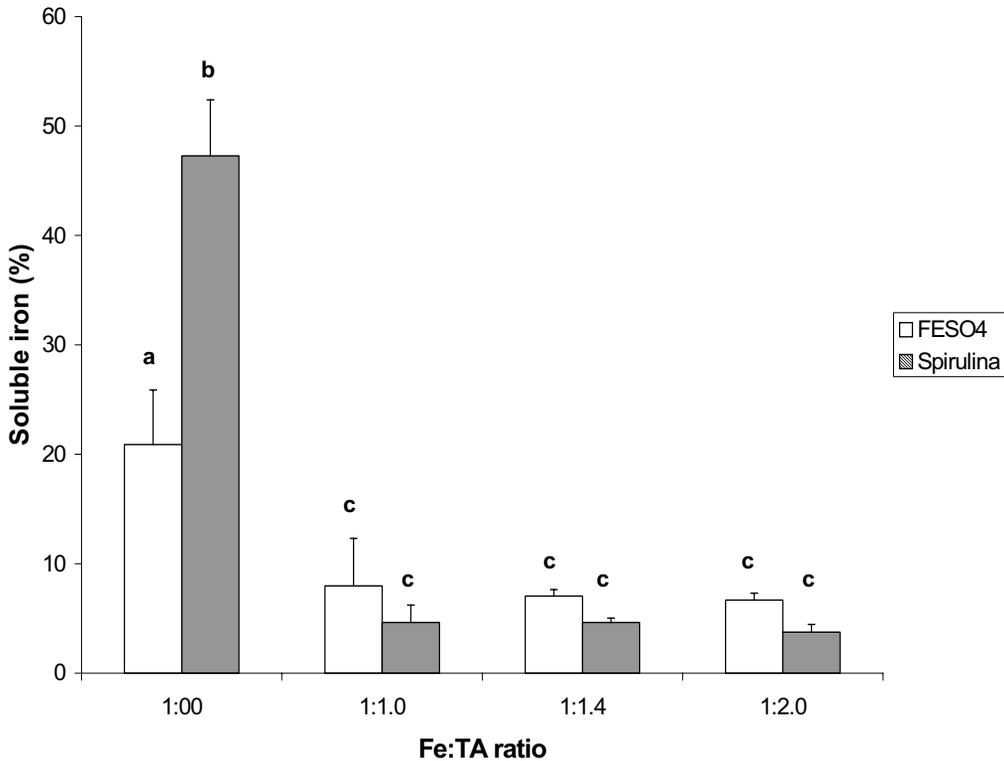
Figure 5. Percentage of soluble iron present after centrifugation at different iron to tannic acid (TA) molar ratios. Values are mean ± SEM, n=3. Bar values with no letters in common are significantly different (P<0.05)

calcium was similar throughout all the molar ratios used. In spirulina, the inhibition was seen less with lower molar ratios as compared with higher molar ratios. The inhibiting effect of calcium was seen higher in $FeSO_4$ group. The reduced iron solubility by calcium might be due to the anion of the calcium salt which could affect iron solubility by way of acid-neutralizing effect as reported by Glahn and co-workers (2000). It is possible that $CaCO_3$ raised the mean pH which might be caused by excess calcium ions in the digest and thus forming $Ca(OH)_2$. An increase in pH of the digesta may lead to

reduced iron solubility (Wienk *et al.*, 1996).

Iron solubility alone does not necessarily predict its uptake or bioavailability. The presence of low concentrations of calcium did not significantly lower iron uptake in $FeSO_4$ and spirulina compared with higher concentrations of calcium which significantly affect the iron uptake from both $FeSO_4$ and spirulina. This indicates a dose effect relationship between the amount of added calcium and degree of inhibition of iron uptake. These findings are consistent with studies by Hallberg *et al* (1991). The iron uptake of spirulina in the presence of low calcium concentra-
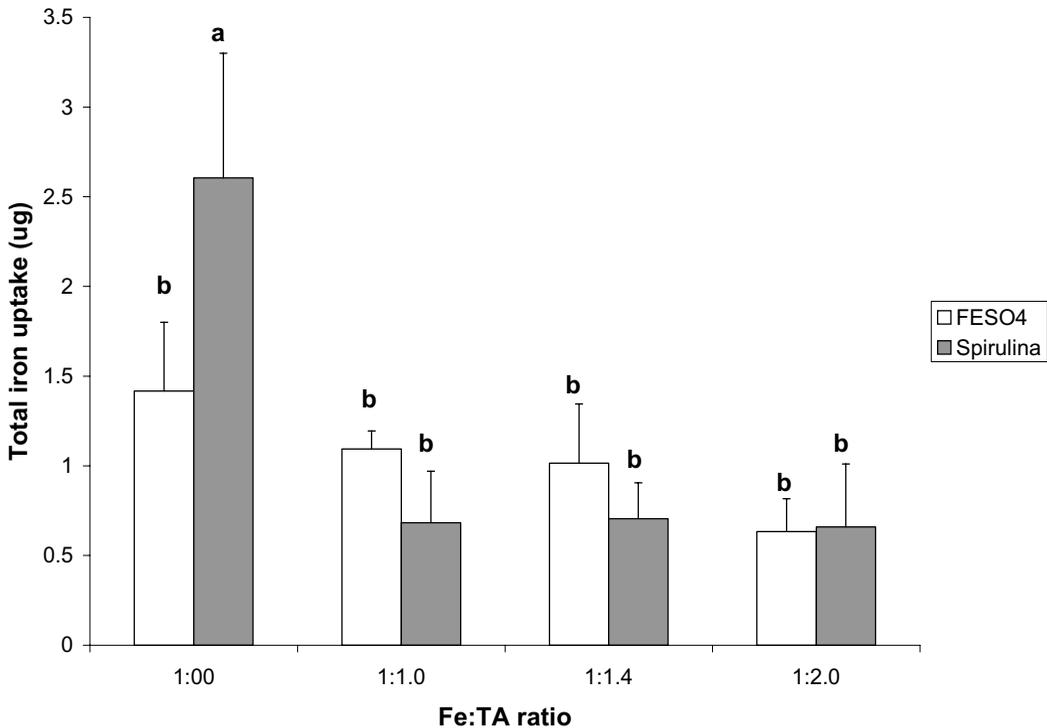
Figure 6. Total iron uptake by Caco-2 cell at different iron to tannic acid (TA) molar ratios. Total iron uptake was calculated by multiplying the % radioiron uptake by iron content in the medium. Values are mean ± SEM, n=3. Bar values with no letters in common are significantly different (P<0.05)

tions (≤ 1:20 Fe:Ca molar ratios) was shown to be higher than that of $FeSO_4$ without any addition of calcium. This indicates that iron in spirulina in the presence of calcium at low concentration was more available than iron from $FeSO_4$ alone. However, at higher concentrations, the iron availability was comparable for both spirulina and $FeSO_4$ as both groups showed inhibition by calcium at this molar ratios. This showed that calcium reduced solubility but did not significantly lower iron uptake at lower molar ratios (≤ 1:20) but did so at higher molar ratios (≥ 1:37). The iron uptake of lower molar ratios was

significantly higher than iron uptake by $FeSO_4$ indicating that even in the presence of low amount of calcium, the iron uptake from spirulina is better than $FeSO_4$.

At low concentrations, calcium does not post a significant inhibition on iron absorption from spirulina but did so in $FeSO_4$. At higher concentrations, the amount was sufficient to inhibit iron uptake by Caco-2 both in spirulina and $FeSO_4$. This indicated that iron from spirulina could compete with calcium of low concentrations for uptake. Hallberg, Rossander-Hulthen & Gleerup, (1992) had suggested that there is competitive inhibi-

tion between calcium and dietary iron sources (haem and nonhaem) within the intestinal mucosal. The location(s) within the enterocyte where calcium may interact with iron uptake remains speculative. The inhibitory mechanism of calcium on iron absorption may involve transfer from the mucosal cell into the circulation (Hallberg *et al.*, 1991; Wienk *et al.*, 1996) or calcium may compete for iron binding sites on the intestinal shuttle protein mobilferrin, which could interfere with intestinal iron uptake (Wolf & Wessling-Resnick, 1994).

There is considerable evidence that supplemental ascorbic acid enhances nonhaem iron absorption in human (Monsen, 1988). Its effect appears to be related to both its reducing power and its chelating action. It can reduce the ferric to ferrous iron and/or maintain ferrous iron in the ferrous state and so prevent or decrease the formation of insoluble complexes with absorption inhibitors or with hydroxide iron in the gut (Hurrell, 1997).

Even though ascorbic acid did not increase iron solubility from spirulina as much as $FeSO_4$, the iron uptake was greatly increased. This showed that iron from spirulina in the presence of ascorbic acid was more efficiently absorbed compared to $FeSO_4$. Studies have shown that dietary constituents such as ascorbic acid do not affect haem iron absorption. Puyfoulhoux *et al* (2001), hypothesised that two forms of iron can be found in spirulina, one containing iron resembling haem iron and another comprising nonhaem iron. The haem iron in spirulina might not be affected by the presence of ascorbic acid and thus the increase in iron uptake was lower compared to $FeSO_4$. Furthermore, spirulina has its own ascorbic acid and may help in maintaining more soluble iron without the addition of ascorbic acid (Fe:AA of 1:0). In this study, the percentage of soluble iron and iron uptake were doubled for spirulina compared to $FeSO_4$.

The presence of tannic acid in a meal has been shown to reduce iron absorption

(Gillooly *et al.*, 1983). The inhibition of iron absorption by tannic acid is likely due to the galloyl groups which have a direct chemical binding effect, especially on ferric iron, and also presumably through the formation of chelates. Polyphenols are thought to act through the formation of complexes between the hydroxyl groups of the phenolic compounds and iron molecules, rendering the iron unavailable for absorption.

In this study, tannic acid significantly reduced iron uptake in spirulina in all molar ratios, while only high molar ratios of tannic acid significantly inhibited iron uptake in $FeSO_4$. The reason might be that tannic acid binds iron from spirulina differently from that of $FeSO_4$. Tannins have the property of precipitating proteins in aqueous media. Spirulina is known to contain high amount of protein (Kay, 1991) and tannic acid might form a strong bond (hydrogen bond) with the basic sidegroups on the protein, and thus preventing the iron from spirulina to be taken up. However, studies by Brune, Rossander & Hallberg (1989) have shown that the interference of tannic acid on iron absorption is not related to the protein-precipitating effect but rather due to a direct complex formation between the phenolic hydroxyls and the iron ions. Tannic acid is hydrolysed in the acidic pH in the stomach and releases the potentially reactive gallic acid residues which bind iron. This might explain the inhibition that occured in $FeSO_4$ especially at higher molar ratios. However in the case of spirulina, both the protein-precipitating effect and the formation of phenolic hydroxyls and iron complex might synergistically affect the iron availability from spirulina in the presence of tannic acid.

Interactions between iron and other food components affect the iron absorption especially nonhaem iron. Although various studies have shown that spirulina is high in iron, however, its bioavailabilty may be inhibited in the presence of

inhibitors such as calcium and tannic acid. The effect of enhancers such as ascorbic acid on iron bioavailability from spirulina was not as high compared to the effect on $FeSO_4$, due to the postulated dual forms of iron in the spirulina. Future studies should be focused on determining the magnitude of effects of these dietary factors on iron bioavailability from spirulina when taken with other foods.

ACKNOWLEDGEMENTS

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