Title: *Iron bioavailability from commercially available iron supplements*

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Abstract

Purpose

Iron deficiency anaemia (IDA) is a global public health problem. Treatment with the standard of care ferrous iron salts may be poorly tolerated leading to noncompliance and ineffective correction of IDA. Employing supplements with higher bioavailability might permit lower doses of iron to be used with fewer side effects, thus improving treatment efficacy. Here we compared the iron bioavailability of ferrous sulphate tablets with alternative commercial iron products, including three liquid based supplements.

Methods

Iron bioavailability was measured using Caco-2 cells with ferritin formation as a surrogate marker for iron uptake. Statistical analysis was performed using one-way ANOVA followed by either Dunnett's or Tukey's multiple comparisons tests.

Results

Spatone Apple® (a naturally iron-rich mineral water with added ascorbate) and Iron Vital F® (a synthetic liquid iron supplement) had the highest iron bioavailability. There was no statistical difference between iron uptake from ferrous sulphate tablets, Spatone® (naturally iron-rich mineral water alone) and Pregnacare Original® (a multi-mineral/vitamin tablet).

Conclusion

In our *in vitro* model naturally iron-rich mineral waters and synthetic liquid iron formulations have equivalent or better bioavailability compared with ferrous iron sulphate tablets. If these results are confirmed *in vivo*, this would mean that at risk groups for IDA could be offered a greater choice of more bioavailable and potentially better tolerated iron preparations.

Key words

Iron supplements, anaemia, pregnancy, bariatric surgery, micronutrient deficiency, Caco-2 cells

Introduction

Iron deficiency is a global health problem; 2 billion people suffer from anaemia and the World Health Organization (WHO) estimates that iron deficiency accounts for 50% of these cases [1]. Although the prevalence of iron deficiency anaemia (IDA) is highest in developing countries, inadequate iron status is also a significant public health problem in developed countries in high-risk groups including the elderly, post-bariatric surgery patients, toddlers, and pregnant women and women of child bearing age [2,3].

Three ferrous iron salts, sulphate, fumarate and gluconate, are the standard recommended supplements for IDA [4,5]. Clinical trials with pregnant women document that these iron supplements do improve IDA [6], however, non-compliance secondary to supplement-associated gastro-intestinal (GI) problems, such as constipation, black stools, nausea, reflux and vomiting, means that real life efficacy may be low [7-9]. Pregnancy itself induces gastrointestinal symptoms, related to the mechanical effects of the growing foetus, changes in water homestasis, and hormonal effects on GI motility - all of which contribute to increased incidence of reflux, constipation and bloating [10,11]. Higher doses of iron increase gastro-intestinal (GI) symptoms in pregnant women [12], and studies document that lower doses are associated with fewer GI symptoms [6,13], thus it is possible that the use of iron preparations with higher bioavailability, enabling lower absolute doses of iron to be ingested, may cause less GI upset.

IDA is associated with increased health risks to both mother and foetus (reviewed in [14]), and it is therefore vital that effective supplement forms are available to pregnant women. A human volunteer study demonstrated that iron absorption from a naturally iron-rich mineral water (Spatone Iron-Plus) was higher compared with ferrous sulphate tablets ($FeSO_4$) [15]. Furthermore, a subsequent study looking specifically at pregnant women with IDA found that the mean absorption from 25 mg

of Spatone derived Fe was 28% - comparable or greater to that reported from a higher dose of $FeSO_4$ [16].

The physical form of iron administered in supplements could have a bearing on bioavailability. A recent *in vitro* study found that dissolution rates varied significantly amongst different iron tablet formulations; $FeSO_4$ conventional-release tablets dissolved most quickly at 48-64 minutes, and this was associated with the highest iron uptake in the *in vitro* Caco-2 cell model used in this study [17]. We hypothesized therefore that liquid iron formulations, whether naturally occurring or synthetic, might provide alternative supplement forms that have higher bioavailability compared with iron delivered in tablet form.

In this study we have employed the Caco-2 cell *in vitro* digestion model to assess iron bioavailability, using cell ferritin as a surrogate marker for iron absorption, from five commercially available iron formulations including three liquid iron supplements [18]. This model is used worldwide to measure iron bioavailability from different substances, and has been validated by human studies [19]. We compared two naturally mineral rich waters, Spatone® and Spatone Apple® (Spatone with added apple flavour and ascorbate), Iron Vital F® (a synthetic liquid iron supplement with added ascorbate and other micronutrients), immediate release $FeSO_4$ tablets, and Vitabiotics Pregnacare® (a multivitamin and mineral tablet marketed to pregnant women).

Materials &Methods

Reagents

Chemicals, hormones and enzymes were purchased from Sigma-Aldrich, UK, unless otherwise noted. Cell culture media, flasks, tissue culture plates and culture reagents were obtained from Thermo Fisher Scientific, and acids used in ICP analysis, digestions and for glassware cleaning were purchased from VWR, UK. Glass and plastic ware used in experiments were soaked in 10% trace metal grade 68% nitric acid for 24 hours prior to use, and then rinsed with 18 mΩ pure water.

Commercial iron preparations were purchased from Boots Pharmacy, UK. These consisted of: Spatone® & Spatone Apple® (A Nelson & Co Ltd, UK); Iron Vital F® (Anton Hubner Gmbh & Co Germany); Pregnacare Original® (Vitabiotics Ltd. UK), and Almus Ferrous Sulphate tablets (Almus Pharmaceuticals, UK). Table one provides iron concentration for the different formulations.

Cell culture

Caco-2 cells (TC7 clone), gifted to the Sharp lab by Monique Rousset and Edith Brot-Laroche [20], were used from cell passages 46-49. The TC7 clone has been validated for use in studies on iron metabolism [21], and has been used in our previous published work on iron bioavailability [22]. Cells were grown in T75 tissue culture flasks and subcultured every 7 days. For experiments, cells were grown in 6 well tissue cultures plates seeded at a density of $1x10^4$ cells/cm^2 and used on days 13-15 post seeding in the method developed by the Glahn lab [18,19]. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, 41965) supplemented with 10% v/v fetal bovine serum (LCG Standards, 30-2020), 1% penicillin-streptomycin, 4 mmol/L L-glutamine, 1% non-essential amino acids, and Plasmocin 5 mg/ml (Source Bioscience).

24 hours prior to the initiation of *in vitro* digestion experiments media was changed to supplemented MEM without foetal bovine serum but with 10 mmol/L PIPES (piperazine-N, N'-bis- [2-ethanesulfonic acid]), 1% antibiotic/ antimycotic solution, 11 mmol/L hydrocortisone, 0.87 mmol/L insulin, 0.02 mmol/L sodium selenite

(Na_2SeO_3), 0.05 mmol/L triiodothyronine and 20 mg/L epidermal growth factor, as developed by Glahn *et al*, in order to ensure adequate cell growth but with low basal media iron levels [18,19].

Iron supplements and in vitro digestions

Iron-containing tablets were sealed inside a disposable plastic sleeve and crushed with a mortar and pestle; the particles were then solubilised in 250 ml 0.2 mol/L hydrochloric acid (HCl) [23]. Liquid formulations were used directly from the sachets (Spatone®and Spatone Apple®), or the bottle (Iron Vital F®). The supplements all had different iron concentrations; therefore, the volumes of prepared solutions, or liquid supplements, were adjusted (based on the manufacturers reported iron concentrations) to achieve a final digest iron concentration of 50 μmol/L.

Fresh tablet solutions were prepared and new sachets were used for every experiment. In addition, all experiments had a set of controls consisting of: a digest with no added iron to ensure no iron contamination of our system; a reference digest of 50 μmol/L Fe added as Fe solubilized in 1% HCl (High-Purity Standards, 100026-2); and a positive control digest of 50 μmol/L Fe and 500 μmol/L ascorbate.

Iron levels of digests were quantitatively analysed by Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES). All iron containing digests, and undiluted samples of Spatone® and Spatone Apple®, were subjected to microwave digestion using an accelerated reaction system (CEM MARS 5H with XP-1500 vessels). 0.5 ml of the solutions (in triplicate) was added to 5.0 ml concentrated 68% trace analysis grade nitric acid. Samples were processed for 20 minutes at 400-psi pressure and 1200-W power. After digestion samples were reconstituted with 50 ml 18 mΩ water and aliquots used to measure iron levels on a Perkin Elmer Optima 4300 DV ICP-OES.

Digests were prepared as previously described [22]. Briefly, iron solutions were added to 10 ml of 140 mmol/L NaCl and 5 mmol/L KCl followed by the sequential addition of pepsin (1 hour in the shaking incubator, pH 2 digests), and then bile and pancreatin digestive enzymes (1 hour in the shaking incubator, pH 7 digests) to mimic the digestive process. 1.5 ml of the above digests was then placed in a chamber

suspended over a layer of Caco-2 cells grown on the bottom of the tissue culture wells of a six well plate. The upper chamber was created using a 15,000 molecular weight cut-off dialysis membrane (Tubing Spectra/Por 7 dialysis membrane, Fisher Scientific) fitted over a Transwell insert and held in place with a silicon ring (Web Seal). Plates were placed on a platform fitted Multi-function 3D rotator (Fisher Scientific PSM3D) set at 6 oscillations per minute in a 37°C incubator with a 5% CO2/95% air atmosphere at constant humidity for 60 minutes. Inserts were then removed and an additional 1 ml of supplemented MEM added to the cells, which were returned to the incubator for a further 22 hours, and then harvested for ferritin. All supplements were tested on three separate occasions, with n=6 for each treatment.

Direct application of iron supplements onto Caco-2 cells to assess dose and treatment duration responses

Caco-2 cells were seeded and maintained as described for *in vitro* digestion experiments. On the day of the experiment fresh solutions of Spatone®, Spatone Apple® and ferric ammonium citrate (FAC) were mixed with plain MEM (herein referred to as MEM) and placed directly onto the cell monolayers at iron concentrations of 10, 30 and 100 μmol/L. In order to assess ferritin formation as a function of treatment duration/time, one set of cells was treated for a full 24 hours, while another set was treated for fours hours (begun 20 hours after media was changed to MEM). At the end of both time periods cells were harvested for ferritin. Experiments were repeated on three separate occasions with n = 6 for each treatment.

Cell harvest and ferritin analysis

Cells were harvested as previously described [22]. Briefly, rinsed cell monolayers were detached with the proprietary cell lysis buffer CelLytic™ (Sigma-Aldrich) with added protease inhibitor. Cells were shaken for 15 minutes on ice on an orbital shaker at 300 rpm, and then spun at 6000 x g for 6 minutes in a 5804R Eppendorf centrifuge. The supernatant was aspirated and stored in a -80°C freezer. For analysis, samples were thawed on ice and ferritin measured with SpectroFerritin MT Enzyme Linked Immunoassay (ELISA; RAMCO). Final ferritin levels were adjusted for cell protein also measured from the supernatants using Pierce Protein BCA Assay (Fisher Scientific, 23227).

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism (v.6.0c GraphPad Software, San Diego, CA). Digest experiments were analysed using the statistical methods of Motulsky [24]. Data are presented as means ± S.E.M and were analysed by one-way ANOVA followed by Tukey's multiple comparisons test (all-pairwise across experimental groups); where the comparison was made to a single control Dunnett's post-hoc test was used. Except as noted differences between means were considered significant at $p \leq 0.05$.

Results

Iron supplements contained different iron concentration (Table 1). Each was prepared to give a notional iron concentration of 50 μmol/L. However, ICP-OES measurements revealed that the iron content of digests varied between different iron sources (Table 2). To adjust Caco-2 cell ferritin levels for the variable iron content of digests we carried out a dose-response study to measure ferritin formation following exposure to different concentrations of iron (Supplementary figure 1). The dose response study demonstrated that ferritin levels in digests were proportional to the log of iron concentration, and thus measured ferritin levels were adjusted using the equation: $F_{adj} = F \times \ln(50)/\ln(Fe_{icp})$ where F_{adj} equals adjusted ferritin, and Fe_{icp} equals ICP measured digest iron levels.

Caco-2 cell ferritin levels were highest in cells exposed to Spatone Apple® (Figure 1). Levels were approximately 500% higher than both Spatone® original and $FeSO_4$ tablets. Iron Vital F® (IVF) induced ferritin formation was approximately 100% higher compared with $FeSO_4$, Spatone®, and Pregnacare Original® (PG) but was only about a third that of Spatone Apple® (Figure 1)**.** Ferritin formation following exposure to Pregnacare Original® was equivalent to that of cells treated with Spatone® and $FeSO_4$.

We further investigated the effects of Spatone® and Spatone Apple® on ferritin levels in Caco-2 cells over a range of iron concentrations, and following different exposure times. Exposure to Spatone Apple® for either 4 h (Figure 2A) or 24 h (Figure 2B) resulted in significantly enhanced ferritin levels at all concentrations compared with the untreated controls. Spatone Apple®-induced ferritin levels were significantly higher than those produced following exposure to iron alone (all concentrations at both 4 h and 24 h time points) and Spatone® original (all concentrations at 24 h; 30 & 100 μmol/L at 4 h).

Discussion

Current strategies for the treatment of IDA are based on oral ferrous iron supplements; however, these are poorly tolerated by patients due to a range of GI side-effects [6-9]. This in turn leads to poor compliance with therapy, which has consequences for the efficacy of treatment. Iron preparations with higher bioavailability, thus enabling lower absolute doses of iron to be ingested, may cause less GI upset and be better tolerated by patients. This notion is supported by a study undertaken in pregnant women who had been non-compliant with taking $FeSO_4$ tablets, and were switched to Spatone iron rich water; 57% of subjects were compliant with the new iron supplement compared with 67% in the controls given placebo (plain water), and dyspepsia scores did not differ between the two groups [25].

We have compared the bioavailability of a range of commercially available iron supplements using a well-characterized *in vitro* digestion model [18,19]. Spatone, and ferrous sulphate tablets had equivalent bioavailability in the *in vitro* model, which is consistent with previous absorption studies in human volunteers [16]. The iron in Spatone® is $FeSO_4$; this suggests that being in liquid form *per se* doesn't increase iron bioavailability. Spatone Apple® demonstrated the highest iron bioavailability in the *in vitro* model, followed by the synthetic liquid iron formulation Iron Vital F®; this result remained highly significant even after adjustment for the increased iron in Spatone Apple® digests. Both Spatone Apple® and Iron Vital F® contain added ascorbate, a known enhancer of iron uptake. While Spatone Apple® has 80 mg ascorbate/sachet (E Hunt, Product & Consumer Information Officer for Nelson & Co., personal communication, 2014) giving an iron:ascorbate molar ratio of 1:16, the iron:ascorbate ratio in Iron Vital F is 1:6 according to the supplement label. The enhancing effects of ascorbate on iron bioavailability are dose dependent [26], therefore the higher ascorbate:iron ratio in Spatone Apple® may explain the difference in iron uptake between the two supplements. Furthermore, Iron Vital F®, according to the manufacturer's ingredients list, also contains plant extracts, pectin and thickening

agents. These dietary factors are sources of polyphenols and phytates, both of which inhibit iron absorption and are only partly counteracted by ascorbate [26]. Interestingly, Vitabiotics Pregnacare® had the same bioavailability as $FeSO_4$, despite having added ascorbate (70 mg/tablet). The iron:ascorbate molar ratio, however, is only 1:4, which may not be optimal for improving iron uptake. In addition, the presence of other minerals contained in Pregnacare®, such as zinc, may partially inhibit iron uptake [27].

Pregnant women are at high risk for IDA [28], however iron supplement recommendations during pregnancy differ between countries. The WHO and the United States of America (USA) recommend that all pregnant women receive prophylactic prenatal iron [29-31]. Currently, the United Kingdom (UK) does not suggest routine prenatal iron supplementation [32]; historically pregnant women in the UK were advised to take iron but this was found to be either ineffective [33], or to have no demonstrated benefit on maternal or foetal outcomes [34,35]. Recent research may challenge current UK guidelines [36]; two large systematic reviews with meta-analyses found that daily prenatal iron reduced maternal anaemia, IDA and risk of foetal low birth weight [37,38]. However, one of the reviews noted that dose and regimen recommendations for routine iron supplementation need refining and updating [37], and within this context our results suggest that further research with the tested formulations used in our study are warranted.

Another group that might benefit from both Spatone Apple® and Iron Vital F® are post-bariatric surgery patients who are at high risk of IDA [39-41], and in whom treatment with standard iron tablets is often ineffective [42,43]. In one study post-operative gastric bypass patients given 100 mg $FeSO_4$ tablets as an oral challenge absorbed inadequate amounts of iron as measured by change in serum iron concentration [44]; altered gut physiology after bariatric surgery may not allow for iron absorption from $FeSO_4$ tablets. Furthermore, several studies have also documented low vitamin C levels in patients after bypass procedures [45,46], therefore liquid iron preparations with added ascorbate - such as Spatone Apple® and Iron Vital F® (which also has other micronutrients) - may be helpful in this population.

The data presented here for Spatone Apple® suggest that this, or similar products, are highly bioavailable as they gave rise to a rapid increase in cell ferritin in our *in vitro* model. Furthermore, 10 μmol/L iron given as Spatone Apple® gave a comparable ferritin response to 100 μmol/L ferric ammonium citrate and a higher ferritin level than 100 μmol/L $FeSO_4$ (from Spatone®). An important caveat is that we have only performed *in vitro* studies to date. The bioavailability of Spatone Apple® has not been assessed in human volunteers, and our data suggest that these studies are warranted. The Caco-2 *in vitro* digestion model provides a validated method for screening iron bioavailability of a range of compounds and test meals. Furthermore, it has been shown to predict the direction, but not necessarily the magnitude, of iron bioavailability in humans [47]. If our *in vitro* results are confirmed *in vivo* it would suggest that lower doses of a more bioavailable iron form could be effective in combating iron deficiency. This would be particularly advantageous for groups who are both at increased risk of IDA and less tolerant to high doses of iron, such as pregnant women.

In conclusion, the results of this *in vitro* study demonstrate that naturally iron-rich mineral waters, or synthetic liquid iron formulations, are equivalent to the standard of care $FeSO_4$ recommended for IDA; and those with added ascorbate have increased bioavailability. If these results are confirmed in randomized control studies in human volunteers at risk for IDA, such subjects could then be offered a greater choice of more bioavailable and potentially better tolerated iron preparations.

Acknowledgments: This work was supported by the Faculty of Engineering & Science at the University of Greenwich, and the Diabetes & Nutritional Sciences Division at King's College London. We thank David Scott Ganis for assistance with statistical and numerical analysis.

The manuscript does not contain clinical studies or patient data.

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Table 1 Manufacturers' information including reported iron content for tested iron preparations

Product name	Reported iron content	Manufacturer
Spatone®	0.25 mg $FeSO_4$/ml	A Nelson & Co Ltd (UK)
Spatone Apple®	0.20 mg $FeSO_4$/ ml	A Nelson & Co Ltd (UK)
Iron Vital F®	1 mg iron-II-gluconate/ml	Anton Hubner Gmbh & Co (Germany)
Pregnacare Original®	51.4 mg ferrous fumarate equivalent to 17 mg ferrous iron/tablet	Vitabiotics Ltd. (UK)
Almus Ferrous Sulphate tablets	200 mg $FeSO_4$ equivalent to 65 mg ferrous iron/tablet	Almus Pharmaceuticals (UK)

Table 2 Iron digest levels and Caco-2 cell ferritin after treatment with iron supplements - unadjusted for iron digest levels

Product name	Digest iron concentrations (mg/L)	ng ferritin/mg protein (unadjusted)
$FeSO_4$	2.80±0.10(0%)	11.82±0.67 (0%)
Iron Vital F®(IVF)	2.90±0.37 (4%)	25.76±2.07 ^{a} (118%)
Pregnacare Original®(PG)	2.83±0.68 (1%)	10.35±0.82 (-12%)
Spatone®	3.40±0.62 (18%)	12.24±0.91 (4%)
Spatone Apple®	4.00±0.75*(30%)	83.94±4.46 ^{b} (610%)

Ferritin levels of Caco-2 cells unadjusted for differing digest iron levels as measured by ICP-OES. Expressed as the mean ± SEM. Means with different superscript letters or asterisk in a column are statistically different. Percentage figures in parenthesis by digest iron levels is the percent by which the mean digest iron levels of other formulations compared with mean $FeSO_4$ iron digest levels; percentage figures in parenthesis by ferritin values are the percent by which the mean ferritin levels of cells treated with the other formulations compared with ferritin of $FeSO_4$ treated cells. Digest iron concentrations for IVF, PG and Spatone®, were not statistically different from that of $FeSO_4$, however digest iron concentrations in Spatone Apple® were significantly different based on one-way ANOVA followed by Tukey's multiple comparison test ($p<0.05$). Ferritin levels were highest in Spatone Apple® treated cells followed by IVF; levels of the three other treatments were not statistically significantly different based on one-way ANOVA followed by Tukey's multiple comparison test ($p<0.05$)

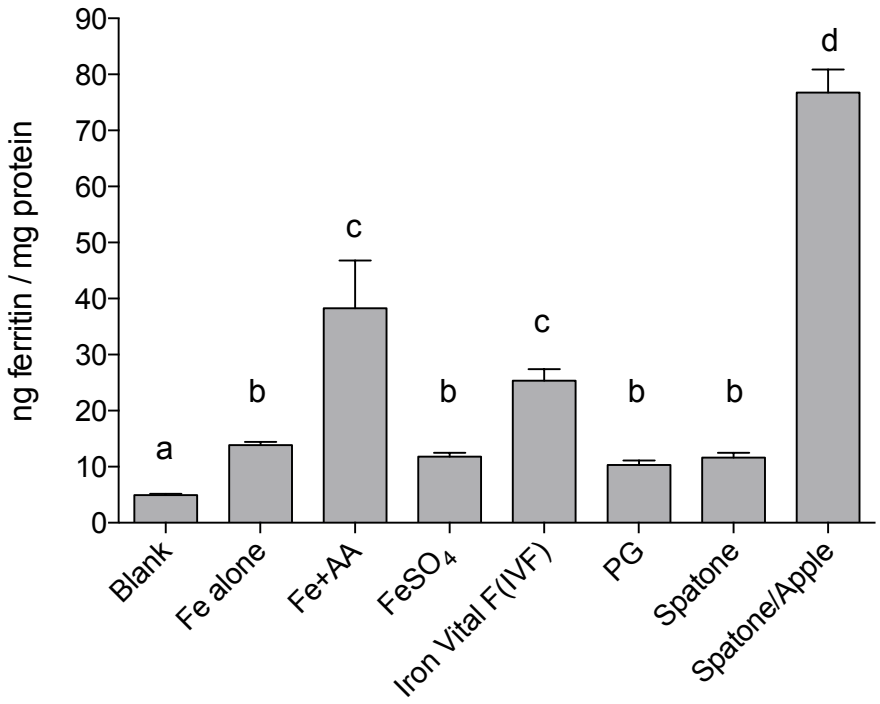


Fig. 1: Comparison of ferritin formation from digests with different commercial iron formulations adjusted for measured iron levels in digests

Measurement of Caco-2 cell ferritin formation from digests of $FeSO_4$ tablets, Iron Vital F® (IVF, a synthetic liquid iron supplement), Vitabiotics Pregnacare® tablets (PG, a mineral and multivitamin supplement marketed to pregnant women), Spatone® and Spatone Apple® (naturally iron-rich mineral waters, the latter with added ascorbate). Digests with no added Fe (Blank) were used to rule out iron contamination; digests with Fe alone and Fe plus ascorbic acid (Fe + AA) were used as reference controls and positive controls, respectively (not unadjusted for digest iron levels). Treatment with Spatone Apple® yielded the highest ferritin levels followed by IVF treatment; ferritin levels from the three other supplements were equivalent. Values are means of data adjusted for the iron content of the digests (as determined by microwave analysis) ± SEM, n=18. Based on an ANOVA ($p<0.0001$) with Tukey's multiple comparisons test post-hoc analysis done on an all-pairwise basis after log adjustment, bar values with no letters in common are significantly different ($p \leq 0.01$)

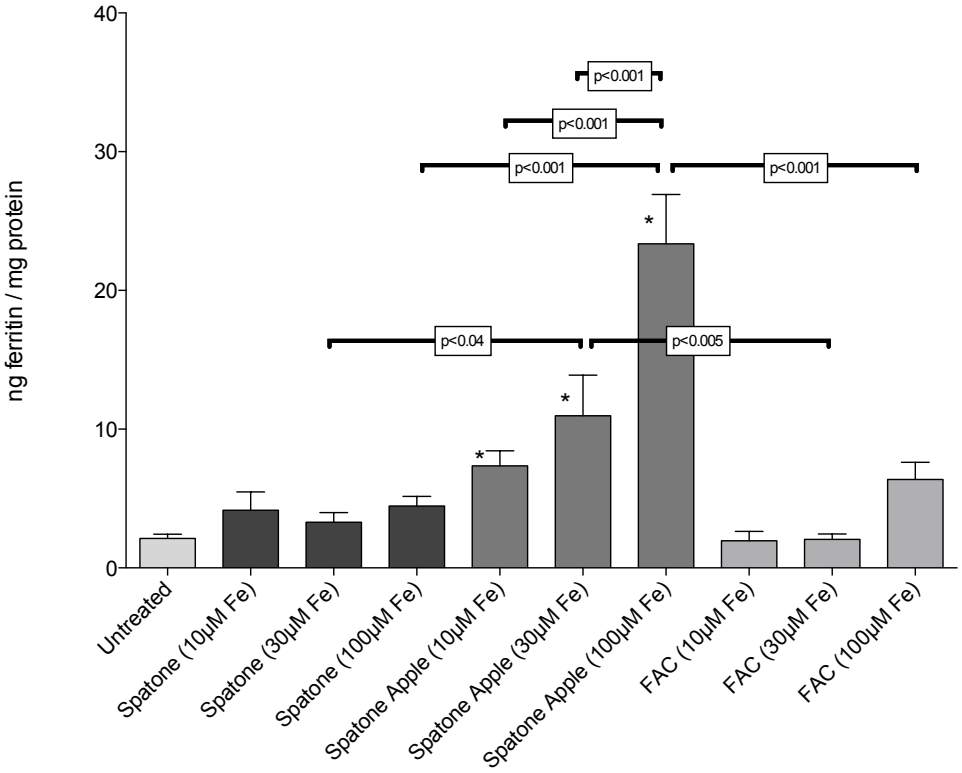


Fig. 2a: Caco-2 cell ferritin levels as a function of iron dose and time – 4 h exposure
Caco-2 cells were treated for four hours with three different concentrations of iron. Ferritin levels increased as a function of concentration with treatments with Spatone® and Spatone Apple®; this only reached statistical significance for Spatone Apple®. Ferritin levels were highest after treatment with Spatone Apple® at all three iron doses tested. Asterisks represent treatments with mean values of ferritin that are significantly greater than the blank control value (labelled untreated) based on Dunnett's multiple comparisons test. Columns connected by lines have significantly different means from one another based on ANOVA followed by Tukey's multiple comparisons test where the Tukey's adjusted p-value is shown within the line ($p \leq 0.05$ is considered significant)

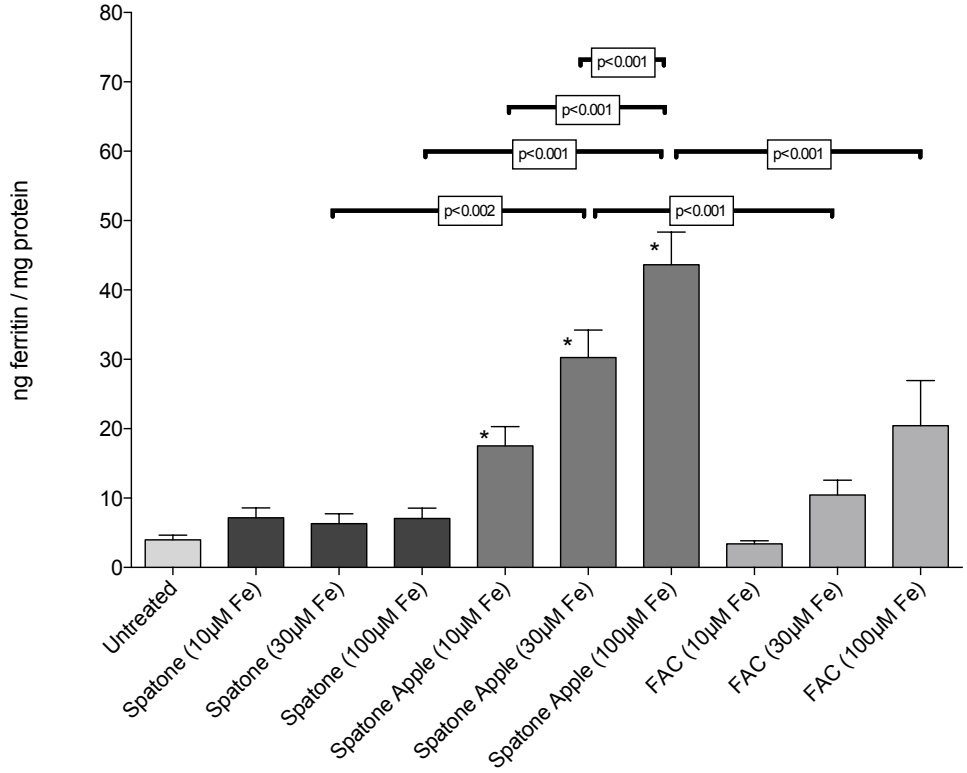
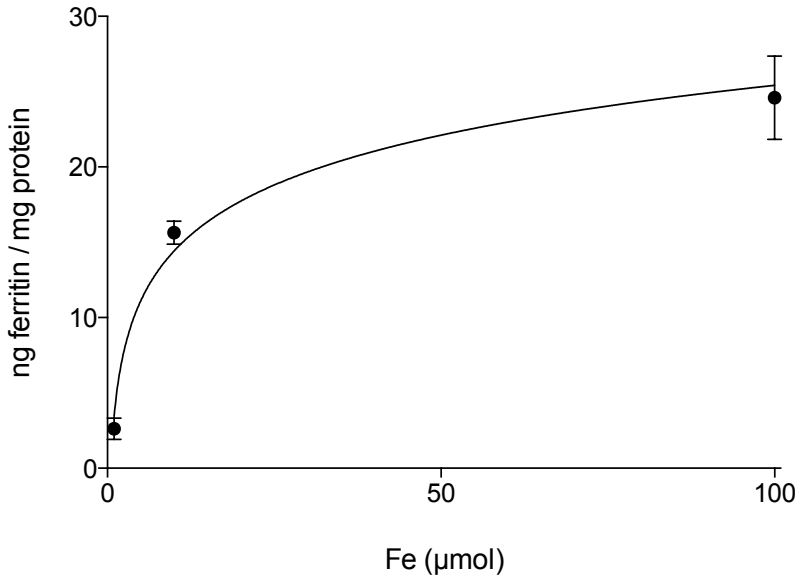


Fig. 2b: Caco-2 cell ferritin levels as a function of iron dose and time – 24 h exposure
Caco-2 cells were treated for twenty-four hours with three different concentrations of iron. Ferritin levels increased as a function of concentration with treatments with Spatone® and Spatone Apple®; this only reached statistical significance for Spatone Apple®. Ferritin levels were highest after treatment with Spatone Apple® at 30 and 100 μmol/L iron. Asterisks represent treatments for which mean values of ferritin are significantly greater than the blank control value (labelled untreated) based on Dunnett's multiple comparisons test. Columns connected by lines have significantly different means from one another based on ANOVA followed by Tukey's multiple comparisons test where the Tukey's adjusted p-value is shown within the line ($p \leq 0.05$ is considered significant)



Supplementary fig.1: Caco-2 cell ferritin levels as a function of iron dose
Caco-2 cells were treated for 24 hours with increasing concentrations of iron ranging from 1-100 µmol/L. Ferritin levels increased as a function of the log of iron concentration