Bioavailability of iron multi-amino acid chelate preparation in mice and human duodenal HuTu 80 cells

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Abstract

Strategies for preventing Fe deficiency include Fe supplementation and Fe fortification of foods. The absorption, metabolism and chemical characteristics of Fe multi-amino acid chelate (IMAAC) are not known. Absorption of IMAAC was compared with FeSO₄ in Fe-depleted mice and in vitro chemical studies of the Fe supplement was performed in HuTu 80 cells. Hb repletion study was carried out in Fe-deficient CD1 mice that were fed for 10 d a diet supplemented with ferrous IMAAC or FeSO₄. A control group of Fe-replete mice was fed a diet with adequate Fe concentrations throughout the study. Tissues were collected from the mice, and the expression of Fe-related genes was determined by quantitative PCR. Ferric reductase and Fe uptake were evaluated in HuTu 80 cells. Supplementation of the diet with FeSO₄ or IMAAC significantly increased Hb levels (P < 0.001) in Fe-deficient mice from initial 93-9 (SD 10-8) or 116-2 (SD 9-1) to 191 (SD 0-7) or 200 (SD 0-5) g/l, respectively. Initial and final Hb for the Fe-deficient control group were 87-4 (SD 6-7) and 111 (SD 11-7) g/l, respectively. Furthermore, the liver non-haem Fe of both supplement groups increased significantly (P < 0.001). IMAAC was more effective at restoring Fe in the spleen compared with FeSO₄ (P < 0.005). Gene expression showed the IMAAC supplement absorption is regulated by the body’s Fe status as it significantly up-regulated hepcidin (P < 0.001) and down-regulated duodenal cytochrome b mRNA (P < 0.005), similar to the effects seen with FeSO₄. A significant proportion of Fe in IMAAC is reduced by ascorbic acid. Fe absorption in mice and cells was similar for both IMAAC and FeSO₄ and both compounds induce and regulate Fe metabolism genes similarly in the maintenance of homeostasis in mice.

Key words: Bioavailability: Iron: Cells: Mice

Fe deficiency anaemia (IDA) is a nutritional disorder afflicting large population groups in the world(1). It is prevalent amongst vulnerable infants, adolescent girls, pregnant women and the aged in both developed and developing countries. The challenge of increased physiological requirements of Fe for growth and reproduction, within these population groups is compounded by inadequate intake and poor Fe bioavailability from foods(2). This is particularly evident in populations subsisting primarily on vegetables or plants for their sources of Fe. Substantive evidence has shown that IDA has debilitating effects on cognition, mental function, work performance and reproductive outcome underlines the need to exploit and explore other sources of Fe as a locus of high concentration of Fe in the gastrointestinal tract(3). Consequently, Fe supplementation and fortification of staple foods in different countries(5) have been practical approaches to alleviate this important nutritional disorder. Fe compounds used for such purposes include ferrous sulphate, ferric pyrophosphate, fumarate, gluconate and ferric ammonium citrate among a host of others. Some of these Fe formulations, because they are redox active, cause irritations or disturbances to the gastrointestinal tract by inducing stomach upset, abdominal pain, constipation or nausea(6–8). Fe supplements, as a locus of high concentration of Fe in the gastrointestinal lumen, could cause DNA damage by generating reactive oxygen species in Fenton reaction. Strategies and initiatives aimed at ameliorating some of the adverse effects of Fe supplements led to the synthesis of nano Fe compounds such as a nano particulate ligand modified Fe(III) polyoxo-hydroxide(9) and nanoparticulate Fe pyrophosphates(10), which were shown to have high bioavailability. This promising outcome underlines the need to exploit and explore other formulations or compounds that exhibit rigid chemical structures, are redox inert, and are highly soluble to maintain colloidal Fe for absorption in the milieu of the gastrointestinal tract. Consequently, Fe multi-amino acid chelate (IMAAC) was formulated and found to be better tolerated than FeSO₄ in a randomised, double-blind placebo-controlled trial (RCT) in healthy premenstrual women(10). There is currently no information on the metabolism and the mechanism of Fe absorption from IMAAC. The present study, therefore, investigates the

Abbreviations: DCYTB, duodenal cytochrome b; IDA, Fe deficiency anaemia; Hb, haemoglobin; IMAAC, Fe deficiency anaemia; RPL 19, ribosomal protein L19.

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bioavailability, characteristics and metabolism of IMAAC using both in vitro cell culture and in vivo mice models. It is hypothesised that Fe absorption from IMAAC will be comparable with that of standard FeSO₄. To test this hypothesis, anaemic mice were administered IMAAC or FeSO₄ to evaluate Hb regeneration, serum, liver and spleen Fe levels and Fe-related genes in the tissues.

Methods

Reagents and chemicals

Unless otherwise stated chemicals and reagents were obtained from Sigma-Aldrich. Standard American Institute of Nutrition diet (AIN-76A) without Fe (low-Fe diet) and the Fe-replete diet contained 48 mg/kg Fe were provided to the mice. IMAAC was prepared and supplied by Biotron Laboratories, Centerville UT, USA. IMAAC used contained 10% elemental Fe chelated in an aqueous solution to low molecular weight peptides, polypeptides and amino acids derived from enzymatically hydrolysed soya protein isolates using non-GM organism Food chemical codex/food grade enzymes. The preparation is then dried and milled to a fine powder. IMAAC preparation with excipients was used in a RCT to test safety and tolerability.

Animal studies

Three-week-old CD1 strain male mice (Charles Rivers) were used for the studies. Mice were housed in a light- and a temperature-controlled room with ad libitum access to diet and water. Fe-deficient diet (TD 80396, 3/kg Fe, and Fe-replete diet TD 80396, 48 mg/kg ferric citrate (Harlan Teklad) were used in the experiment and the diets composition, a modification of AIN-76A is given by Chaudhury et al.

Hb repletion study

In all, fifteen CD1 mice were made Fe deficient on a low Fe diet of 3 mg/kg, for 3 weeks. Five mice were also placed on a normal Fe-replete diet (48 mg/kg) to serve as a control. Following this, blood was withdrawn from the tails to determine the initial Hb levels of the mice. The Fe-depleted mice were then divided into three treatment groups based on similar Hb levels. These fifteen mice were maintained on the low-Fe diet in groups of five, of which one group did not receive any Fe supplementation (low-Fe diet), the two other groups were gavaged daily liquid solutions of 150 µg of Fe as IMAAC or FeSO₄. This was done for 10 d, and the total food consumption of the mice was measured. After 10 d of Fe supplementation, the mice were weighed, anaesthetised and blood samples were taken for Hb and serum Fe determinations. The mice were then killed, and the spleen, duodenum, kidney and liver samples were excised, snap frozen in liquid N₂ and stored in −80°C for further analysis. The experiment was approved by the Institutional (King's College London) Animal Welfare and Ethics Board. All procedures were approved and conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Measurement of Hb, serum iron and tissue non-haem Fe contents

Hb concentrations were calculated from the change in optical density at 540 nm, following the addition of 5 µl of whole blood to Drabkin's reagent (Sigma-Aldrich) and centrifugation (Heraeus Biofuge Pico) at 13 000 rpm for 5 min. Serum Fe was measured with a liquid ferrozine-based Fe reagent (Thermo Electron).

Tissue non-haem iron. Tissue samples were weighed and homogenised (1:5, w/v) in 0-15 m-NaCl in 10 m-NaOH-Hepes buffer (pH 7.0) using a 1 ml glass Dounce homogenizer (Wheaton Scientific). An aliquot of the homogenate was then analysed for non-haem Fe content as described by Simpson & Peters. The Fe values were expressed as either content (µmol Fe/organ) or concentration (nmol Fe/mg wet weight).

Quantitative real-time reverse transcription PCR

Total RNA was extracted from tissue samples using TRIZOL reagent (Invitrogen) according to manufacturer's instructions. Quantitative RT-PCR was carried out using an ABI Prism 7000 (Applied Biosystems) detection system in a two-step protocol with Roche Universal primers and probes. Quantitative measurement of each gene was normalised to the cycle threshold value for ribosomal protein L19 (ribosomal protein L19).

Sequences of mouse primers used, forward and reverse, respectively, are as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dcytb</td>
<td>GTGACCAGCTCTCGTCTTC</td>
<td>TGGATGATTTTCTTCAAGAGC</td>
</tr>
<tr>
<td>Hepcidin</td>
<td>AGAAAGCAAGGAGCACATGG</td>
<td>CACCTGGAATATGTACAGCAATTT</td>
</tr>
<tr>
<td>DMTI</td>
<td>CACCGTACATCTCCAAGGT</td>
<td>CCAATGATGGCCAACCTCCA</td>
</tr>
<tr>
<td>Ferroportin</td>
<td>TTGTTGTTGTGGCAGAGAA</td>
<td>ACGTGATCTACTCTTATAAG</td>
</tr>
<tr>
<td>RPL 19</td>
<td>CTGCAGTACCTCTTCTCA</td>
<td>TCATCCAGGTACCTTCTCA</td>
</tr>
</tbody>
</table>

Cell studies

Duodenal HuTu 80 cells were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% fetal calf serum (Sigma-Aldrich) and with 100 µg/l of penicillin and 100 µg/l streptomycin. Cells were maintained at 37°C in an atmosphere of 5% CO₂ and 95% air at a relative humidity of approximately 95%. Cells were passaged at 70% confluence using Gibco Versene Solution (Life Technologies).

Iron uptake. A balanced salt solution (BSS) containing 130 mm-NaCl, 10 mm-KCl, 1 mm-MgSO₄, 5 mm-glucose and 1·8 mm-CaCl₂ in 10 mm-piperazine-N,N’-bis(2-ethanesulfonic acid) buffer (pH 6·5) that was reported to prevent precipitation or aggregation of
nano-Fe(II), Fe compound was used for cell uptake studies\(^{(15)}\). Unless otherwise stated the Fe concentration was 10 µM. Confluent HuTu 80 cells were exposed to serum-free DMEM for 4 h before Fe uptake studies. The cells were washed with pre-warmed PBS before incubation with the different Fe compounds or the non-supplemented BSS control for 1 h at 37°C. After the Fe incubation period, the uptake medium was discarded, and cells were washed three times with PBS–EDTA (2 ms) to remove Fe that bound non-specifically to the cell membrane. Cells were suspended in fresh serum-free DMEM and incubated for a further 23 h for ferritin synthesis. Following this incubation period, cells were washed with PBS and lysed with Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). The cell lysate was centrifuged (5 min, 16,000 g) to remove cell debris and the supernatant used for ferritin and protein analysis.

**Ferritin ELISA assay in cell lysates.** The Spectro Ferritin MT ELISA Kit (ATI Atlas) was used to determine cellular ferritin content as described in the manufacturer’s protocol. Cellular protein concentration was determined according to Bio-Rad assay protocol (Bio-Rad Laboratories). Experiments were carried out in triplicates, and data are expressed as ng ferritin per mg protein.

Fe uptake and the effect of ferrous Fe inhibition were determined in cells by co-incubating ferrozine (1 mM) with 20µM Fe from IMAAC and FeSO\(_4\) for 1 h. Following this, cells were washed with versene (PBS–EDTA solution) and incubated with serum-free DMEM for 23 h to allow ferritin synthesis.

**Ferric reductase assay**

Ferric reductase assay was performed on HuTu 80 cells plated into twelve well plates. The cells were washed three times with PBS, before incubation in a BSS buffer supplemented with either IMAAC or ferric citrate (different concentrations) and 200µM 3-(2-pyridyl)-5,6-diphenyl 1,2,4-triazine – 40 400-disulfonic acid sodium salt (ferrozine). Ferric reduction of Fe was measured by the formation of the coloured Fe(II)-ferrozine complex and monitoring the change in absorbance at 562 nm.

**Statistical analysis**

All values are expressed as means with their standard errors from at least three different experiments. Statistical differences between means were calculated using Student’s t test in correcting for differences in sample variance. When multiple comparisons were necessary, one-way or two-way ANOVA was performed, using GraphPad with Tukey’s post hoc test. Differences were considered significant at \( P < 0.05 \).

### Results

**In vivo bioavailability of Fe multi-amino acid chelate in a mouse feeding study**

There were no statistical differences \((P > 0.05)\) in initial and final body weights between the experimental groups over the intervention period (Table 1). Average feed intake was 71.76 g and there were no differences between the groups (Table 1). Fe intakes of mice expressed as mg/kg body weight are, respectively, 0.62 (SD 0.02), 4.27 (SD 0.11), 4.54 (SD 0.17) and 9.88 (SD 0.33) for Fe-deficient, FeSO\(_4\), IMAAC and Fe-replete diets.

Efficacy of Hb repletion was employed to compare IMAAC and FeSO\(_4\) following diet-induced Fe deficiency. Mice fed a diet low in Fe (Fe-deficient group) for 4 weeks showed significantly \((P < 0.001)\) lower blood Hb levels than control mice kept on the Fe-replete diet (Fe-replete group) throughout (Fig. 1(a)). Supplementation of the Fe-deficient diet with either IMAAC or FeSO\(_4\) for 10-d significantly increased Hb levels in comparison with mice fed the Fe-deficient diet throughout \((P < 0.001)\) and \((P < 0.005)\), respectively (Fig. 1(a)). Furthermore, Fig. 1(b) shows that only supplemented mice had a significant gain in Hb levels from baseline. Moreover, serum Fe levels were significantly higher in the mice fed the Fe-replete diet \((P < 0.005)\) or supplemented Fe \((P < 0.001)\) than the mice on the Fe-deficient diet (Fig. 1(c)). There was no significant difference in serum Fe between mice, which were given FeSO\(_4\) or IMAAC supplementation \((P > 0.05)\).

**Tissue non-haem iron of mice**

Mice receiving Fe supplementation had significantly higher liver non-haem Fe levels (Fig. 2(a)) than the mice receiving the Fe-replete diet \((P < 0.005)\) and the Fe-deficient diet \((P < 0.001)\). Mice on the control Fe-replete diet had significantly higher liver Fe levels that the Fe-deficient group \((P < 0.05)\) as expected. There was no difference in liver levels of Fe between FeSO\(_4\) and IMAAC supplementation \((P > 0.05)\). Similarly, mice on Fe-replete or FeSO\(_4\) or IMAAC supplementation exhibited significantly higher \((P < 0.001)\) splenic Fe levels than mice fed

### Table 1. Initial and final body weights and feed intake of the experimental groups* (Mean values and standard deviations, \( n \) 5)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fe-replete</th>
<th>Fe-deficient</th>
<th>FeSO(_4)</th>
<th>IMAAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>34.04 ± 1.9</td>
<td>32.17 ± 2.4</td>
<td>34.19 ± 2.3</td>
<td>32.60 ± 2.8</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>34.85 ± 2.7</td>
<td>33.74 ± 1.9</td>
<td>35.24 ± 2.0</td>
<td>33.27 ± 3.0</td>
</tr>
<tr>
<td>Feed intake (g)/10 d</td>
<td>71.38 ± 0.8</td>
<td>71.43 ± 1.5</td>
<td>72.45 ± 1.6</td>
<td>71.78 ± 0.9</td>
</tr>
</tbody>
</table>

IMAAC, Fe multi-amino acid chelate.

* Data were analysed by two-way ANOVA followed by the Tukey’s post hoc test. There were no significant differences in the parameters between the groups.
Fe-deficient diet (Fig. 2(b)). As shown in Fig. 2(c), although the duodenal Fe levels increased in mice fed FeSO₄ or IMAAC supplements, the difference only reached significance level ($P<0.05$) in the mice on the replete Fe diet compared with mice on the Fe-deficient diet. This trend is similar to the observation in the kidney as shown in Fig. 2(d).

Fig. 1. Intragastric administration of iron multi-amino acid chelate (IMAAC) or FeSO₄ and haematic responses of anaemic mice. Hb levels of male CD1 mice following 10-d of administering the test diets (a), Hb gains (b) and serum iron levels in the mice (c). Iron-replete (□) control diet contained 48 mg Fe/kg diet; the iron-deficient (■) diet contained approximately 3 mg Fe/kg diet. Mice were maintained on the iron-deficient diet and were supplemented daily by oral gavage with 150 µg Fe as IMAAC (□) or FeSO₄ (■). Values are means (n 5), with their standard errors. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Fig. 2. Intragastric administration of iron multi-amino acid chelate (IMAAC) or FeSO₄ and tissue iron distribution of anaemic mice. Non-haem iron levels in the liver (a), spleen (b) duodenum (c) and kidney (d) of male CD1 mice following 10-d feeding with different test diets. Iron-replete (□) control diet contained 48 mg Fe/kg diet; the iron-deficient (■) diet contained approximately 3 mg Fe/kg diet. Mice were maintained on the Fe-deficient diet and were supplemented daily by oral gavage with 150 µg Fe as IMAAC (□) or FeSO₄ (■). Values are means (n 5), with their standard errors. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. 

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Fig. 3. Intragastric administration of iron multi-amino acid chelate (IMAAC) or FeSO4 and mRNA levels of iron metabolism genes of anaemic mice. Hepcidin mRNA levels in the liver (a) of male CD1 mice following 10-d feeding with different test diets as described above. Dcytb mRNA expression (b), DMT1 (c) and ferroportin (Fpn) (d) levels in the duodenum of mice on experimental diets. , iron replete; □, iron deficient; ■, FeSO4; ▲, IMAAC. * P < 0.05, ** P < 0.01, *** P < 0.001.

Gene expression in the duodenum and liver tissues of mice

As expected Fig. 3(a) indicates that hepcidin mRNA expression in the liver was up-regulated significantly in animals fed Fe replete and supplemented Fe and significantly down-regulated in animals on the Fe-deficient diet (P<0.001). Consequently, duodenal cytochrome b (Dcytb) mRNA levels (Fig. 3(b)) increased in the duodenum of the mice given the Fe-deficient diet. Moreover, a trend of increase was observed in the duodenal levels of divalent metal transporter 1 (DMT1) and Fpn mRNA of Fe-deficient mice (Fig. 3(c) and (d)). Fe supplementation, however, significantly (P<0.001) down-regulated Dcytb mRNA levels in the duodenum of mice. Down-regulation of Fe transport genes was more responsive to FeSO4 than IMAAC administration (Fig. 3(c) and (d)). Ascorbic acid significantly enhanced (P<0.01) ferric reduction in both Fe compounds.

In vivo iron uptake and ferric iron reduction studies in HuTu 80 cells

Consistent with the observation in the in vivo mouse studies, IMAAC and FeSO4 exhibited comparable Fe uptake values in HuTu 80 cells (Fig. 4(a)). However, Fe(II) chelator ferrozine, significantly inhibited Fe uptake and utilisation from FeSO4 (P<0.05), the effect on IMAAC was not significant (Fig. 4(b)). Ferric citrate reduction in HuTu 80 cells exhibited pH-dependent activity that is higher than that of IMAAC (Fig. 4(c) and (d)). Reductase activity was significantly different (P<0.001) between pH 5-5 and 7-4 for both ferric citrate and IMAAC. However, reduction of Fe and dissolution in the presence of ascorbic acid was higher with IMAAC (Fig. 4(c) and (d)). Ascorbic acid significantly enhanced (P<0.01) ferric reduction in both Fe compounds.

Discussion

Fe supplementation and food fortification are panaceas aimed at improving Fe nutrition(14,15) particularly to the vulnerable groups of the population. These strategies, however, are associated with diverse issues limiting effectiveness and as such are subjects of continuing research and investigations. FeSO4, the standard Fe supplement is highly reactive, potentially toxic and prone to causing gastrointestinal disturbances. Alternatives such as IMAAC was reported to be better tolerated than FeSO4 in a RCT in healthy premenstrual women(10). The bioavailability, metabolism and the mechanism of Fe absorption from IMAAC were consequently investigated in Fe-deficient mice in the present study. In general, studies in humans revealed that differences in diets that vary in bioavailability are apparent in Fe-deficient rather than in Fe-replete subjects(16).

The data demonstrate the comparable efficiency of both IMAAC and ferrous sulphate in replenishing Hb in Fe-deficient mice and similar Fe uptake in vitro in intestinal HuTu 80 cells. This agrees with the observation of which Fe glycine chelate compared with FeSO4 in Hb repletion of chicks(17) and in a study that examines ferrous carbamoyl glycine with FeSO4 in rats(18). Furthermore, Fe glycine chelate was equally efficacious in treating cancer patients with mild IDA(19). Remarkably, however, IMAAC is a repository of Fe and several amino acids. Consequently, tolerability of IMAAC possibly accrues from combined antioxidant properties of the constituent multiple amino acids(20).
The study next compared the metabolism of Fe in IMAAC with FeSO₄ in mice. During Fe deficiency, liver and spleen Fe stores are rapidly depleted to maintain Fe homoeostasis and functional Fe requirements. Consequently, repletion of these tissues during Fe supplementation is a relative marker of bioavailability. IMAAC and FeSO₄ supplementation had equivalent levels of liver Fe repletion (Fig. 2(a)) compared with the Fe-deficient diet (P<0·001). Furthermore, in the spleen (Fig. 2(b)) the level of non-haem Fe was higher in the mice given IMAAC (P<0·005) than FeSO₄ compared with the Fe-deficient diet. In fact, FeSO₄ caused a similar level of splenic non-haem Fe to the Fe-replete diet. The spleen is the last tissue in the body to replenish its Fe stores after Fe deficiency[21]. This observation was also evident in pregnant pigs that were given hydrolysed soya protein Fe AAC supplement for 4 weeks before parturition. On analysis, there was 34·5 % greater non-haem Fe levels in the liver and 8·5 % higher Fe in the spleen of piglets whose mothers were given the Fe amino acid chelate supplement compared with the control group[22]. In addition to tissue Fe, IMAAC modulation of Fe metabolism genes in mice was also evident in the present study.

Remarkably, though, administration of IMAAC supplement for 10 d replenished Fe stores of anaemic mice to the same level as the normal healthy mice kept on the Fe replete diet despite significant difference (P<0·001) in Fe intake (4·54 (sd 0·17) v. 9·88 (sd 0·35) mg/kg body weight). Fe intake (4·27 (sd 0·11) mg/kg body weight) of mice that were given FeSO₄ was comparable with those on IMAAC. This attests to the superior Fe bioavailability of IMAAC and FeSO₄ than ferric citrate Fe component of the Fe-replete diet (TD 80396, 48 mg/kg ferric citrate (Harlan Teklad)). Contrary to some concerns[23] that Fe chelates if absorbed intact could bypass the normal homoeostatic regulatory mechanisms, Fe amino acid chelates were reported to be regulated by the body’s Fe status in humans[24]. Expression of hepcidin and other Fe metabolism genes were mostly appropriately modulated by IMAAC and FeSO₄ supplementation in the present study. As expected from gene expression studies, hepcidin mRNA levels (Fig. 3(a)) was down-regulated in mice given the Fe-deficient diet and up-regulated by both Fe supplements (P<0·005). Consequently, DMT1, ferroportin and, in particular, Dcytb mRNA levels (Fig. 3(b)–(d)) were up-regulated in mice on the Fe-deficient diet and down-regulated in both groups on supplements (P<0·001). These results reflect the effectiveness of both Fe supplements in resolving IDA. Furthermore, increased hepcidin expression will exert a negative feedback regulation of the genes involved in Fe transport machinery until Fe homoeostasis is restored. Hb repletion and hepatic hepcidin mRNA levels exhibited a trend that reflected responsiveness to bioavailability of IMAAC and FeSO₄ in mice (Figs. 1(a) and 3(a)). This might be similar to the luminal endocytic uptake of nanoparticulate Fe(3+) poly oxo-hydroxide (nanoFe(3+)3), which differs from the uptake mechanism of inorganic ferrous ion[25], but both share a ferroportin-dependent abluminal efflux mechanism into the circulation[25]. This is consistent with the concept of the labile Fe
pool in the cytosol, where Fe is released from inorganic or organic complexes into the common matrix for efflux into circulation as regulated by ferroportin/hepcidin interaction.

The chemical nature of IMAAC and the mechanism of the Fe uptake process were studied in duodenal HuTu 80 cells (Fig. 4(a)). The data suggest that the content of ferrous chelatable Fe is more negligible in IMAAC than FeSO₄ (Fig. 4(b)). Food Fe sources and Fe salts are liable to autoxidation to the ferric form as they traverse the neutral pH section of the gastrointestinal tract. Consequently, the rate of dissolution of Fe in different formulations is critical in the absorption efficiency. We next investigated the reductive dissolution of IMAAC in the presence of ascorbic acid and compared this to ferric citrate. While ferric citrate exhibited a pH-dependent reductive dissolution of Fe (Fig. 4(c) and (d)), the dissolution of IMAAC seems independent of pH. Ferrozine chelable Fe was higher for FeSO₄ than that of IMAAC, thus implying the availability of less redox active species in the later. This perhaps explains the reported higher tolerability of IMAAC over FeSO₄ in a RCT in human subjects. However, negligible free ferric Fe component of IMAAC could be easily reduced by ascorbic acid (Fig. 4(c)). Chelation, sequestration and encapsulation of Fe by amino acids and peptides possibly prevent redox reactivity in the lumen of the gastrointestinal tract. For inorganic Fe salts, however, the rate of autoxidation and dissolution of the ferric Fe in different formulations could be critical in the absorption efficiency in the proximal duodenal tract. However, the resultant consequences on microbial population and the generation of reactive Fe species in the distal duodenum are subject of continuing investigation.

IMAAC has been shown to compare favourably well with FeSO₄ in Hb regeneration of anaemic mice and in vitro HuTu 80 intestinal Fe uptake studies. It has potential, therefore, to be used widely as an alternative to FeSO₄ in the management of IDA as it has been shown to be better tolerated than FeSO₄ in humans. In vitro cell culture studies offer opportunities for screening Fe compounds such as pyrophosphate, gluconate, ammonium citrate and bis glycinate, etc. as the relative stability of various Fe compounds in vivo has been a matter of uncertainty to the nutrition industry. On the other hand, animal studies may be useful for evaluating relative bioavailability and understanding the mechanisms of Fe absorption; the results may not wholly translate to what occurs in humans and interpretations as such should be treated with caution. Future studies on IMAAC could be for a longer duration to investigate the balance of the highly bioavailable Fe species and the potentials of pathogenic microbes to pilfer this Fe source for proliferation in the lower segment of the gastrointestinal tract. Fe absorption in mice and cells was comparable for both IMAAC and FeSO₄ and both compounds induced and regulated Fe metabolism genes in a similar way.

Acknowledgements

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G. O. L.-D. designed and conducted the research, analysed the data and wrote the manuscript; N. K., C. B., A. C. and G. T. performed the experiments, data collection and statistical analysis. All authors declare that they have no conflicts of interest.

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