Development of the Caco-2 Model for Assessment of Iron Absorption and Utilisation at Supplemental Levels

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Abstract: Caco-2 cells may be typically used as a first step to investigate the bioavailability of different dietary and fortificant forms of iron (Fe) at low levels (< 10 μM) in tissue culture medium (TCM). Whether this model is suitable with supplemental levels of Fe (ca. 200 μM in TCM) is not clear and neither, therefore, is the choice of reference iron compound under those conditions as a 'positive control'. Here we show that with 200 μM iron in TCM (serum-free MEM), Fe(II) sulphate precipitates and while high levels of ascorbic acid can prevent this, it is to the detriment of the Caco-2 cell monolayer and/or it adversely affects the pH of the TCM. Adjusting the pH of TCM to account for this issue again leads to Fe precipitation, which is detectable as both a true precipitate (~ 50%) and a nano-precipitate in suspension (~20%). In contrast, Fe(III) maltol which, clinically, appears less toxic to the intestinal mucosa than Fe(II) sulphate, retains solubility at supplemental levels in cell culture medium, without adversely affecting pH or the Caco-2 cell monolayer. Moreover, the iron is also well utilized by the cells as assessed through ferritin formation. Thus Caco-2 cells may also provide a model for screening iron uptake and utilisation at supplemental levels through the cellular generation of ferritin although care must be taken in ensuring (i) appropriate TCM conditions (e.g. pH and chemical speciation of the iron) (ii) monolayer integrity (i.e. the assay response is not an artefact of toxicity) and (iii) that an appropriate reference material is used (e.g. Fe:maltol at 1:5 ratio).

Keywords: Ferrous sulphate, soluble iron, Caco-2, TEER, ferric maltol, iron supplementation, ferritin formation, iron uptake, iron utilisation.

INTRODUCTION

The average daily intake of non-haem dietary iron in the Western world is around 10 mg/adult and the postprandial, duodenal concentration of ionisable iron is probably ca. 10 μM [1]. In contrast, with therapeutic oral iron supplementation, the duodenal iron concentration is likely to be ca. 200 μM, assuming a dose of 60mg Fe and allowing for the dilution effect in the stomach and duodenum [2-4]. The Caco-2 cell model is well established for studying iron absorption at typical dietary levels [5, 6] but has not been investigated for use with these 20-fold higher, supplemental levels of iron. The current unmet need for a cheap, efficacious and safe oral iron therapy underlies the importance of establishing a cellular assay to screen potential candidate compounds. The 'positive control' for such an assay is not obvious: ferric salts would be insoluble at the peri-neutral pH of tissue culture medium (TCM) whilst simple ferrous salts are also unlikely to be soluble at these higher iron levels. The commonly used 'iron ascorbate' system is also complicated at higher doses of iron in TCM: whether Fe(III) or Fe(II) salts are prepared with ascorbate, a reduced iron (i.e. Fe(II) complex) will be formed. However, pH (i.e. $[OH^-]$) relative to [Fe] and [ascorbate] critically determines overall solubility [7]. Thus the ratio of ascorbate:iron that is required at dietary levels of iron in TCM [8, 9] will be lower than the ratio required at therapeutic (i.e. supplemental) iron levels. By increasing media levels of ascorbate, cellular toxicity could ensue [10-12] although whether iron binding would attenuate or intensify this is not clear. This work focuses on how/whether the Caco2 gut-cell assay can be used to assess supplemental iron utilisation and, if so, what the best positive control would be. In order to use the Caco-2 model for these uptake studies, the formation of an intact monolayer is crucial as this mimics the epithelial barrier *in vivo*. Trans-epithelial electrical resistance (TEER) measurements are often employed to assess monolayer integrity during the testing of different experimental conditions and are a good indicator of any damage or undesired tight junction permeability arising from those conditions [13-15].

Hence in this work, we have sought iron preparations that remain soluble in TCM and available to the cell but, also, that do not disrupt the cell monolayer, all at supplemental levels.

MATERIALS AND METHODS

Reagents

All chemicals and reagents were obtained from Sigma-Aldrich (Dorset, UK) unless otherwise stated. All

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solutions were prepared in 18.2 MΩcm^{-1} ultrapure water (H_2O_{UHP}).

Preparation of Soluble Ferrous Iron Solutions

Immediately before the experiments, an acidic (pH 1.5) stock solution of Fe(II) [ferrous] sulphate ($FeSO_4$) in H_2O_{UHP} (Fe concentration = 40 mM) was combined with an ascorbic acid stock solution in MEM (Minimum Essential Medium, pH 7.4, PAA Laboratories, Yeovil, UK) (ascorbic acid concentration = 0.5M) to obtain the different ratios of Fe to ascorbic acid. Ascorbic acid is readily oxidised by oxygen and it is recommended to be prepared fresh for experiments. The final Fe concentration was 200 µM throughout and the ascorbic acid concentration ranged from 0 to 200 mM. The resulting solutions were filter sterilized (0.2 µm syringe filter). and kept protected from light for the duration of the experiments.

Iron(III) [ferric] maltol (FeM) was prepared by mixing an 8 mM solution of $FeCl_3$ in H_2O_{UHP} with a 40 mM maltol solution to achieve a molar ratio of metal:ligand of 1:5 and for which with simple mixing at these concentrations iron solubility of the complex was found to be optimal without maltol excess (data not shown). The pH of the mixture was adjusted to 7.4 with NaOH, the solution was filter-sterilized (0.2 µm syringe filter) and diluted in the cell culture medium to a final Fe concentration of 200 µM.

The total iron content was determined in the Fe containing experimental solutions by inductively-coupled plasma optical emission spectrometry (ICP-OES Jobin Yvon JY 2000; Horiba Jobin Yvon Ltd, Stanmore, UK). Both ICP-OES standards and samples were diluted in 0.5 % HNO_3 to concentrations in the range 0-1000 ppb (parts per billion). Additionally, the different preparations were characterized in terms of iron phase distribution in the cell culture medium. To distinguish between soluble and particulate iron, Fe solutions were centrifuged (10,000 *xg*, 5 min) and the supernatant was analysed for Fe content. Fe found in this fraction should be free of large agglomerates and contains therefore only nanoparticulate (colloidal) and soluble iron. Further ultrafiltration through a 3 kDa MWCO filter (10,000 *xg*, 10 min) allows the determination of the soluble iron fraction (ca. < 1 nm). The nanoparticulate Fe fraction was then calculated by subtracting the soluble Fe from the Fe in the supernatant. All fractions were then expressed as percentages ± SD in relation to total Fe content. These results were calculated as follows:

[(%) microparticulate] = [(total Fe – $Fe_{supernatant}$)/total Fe] x 100

[(%) nanoparticulate] = [($Fe_{supernatant}$ – $Fe_{ultrafiltrate}$)/total Fe] x 100

[(%) soluble] = [($Fe_{ultrafiltrate}$)/total Fe] x 100

Cell Culture

Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) at passage 18 and were cultured in MEM (PAA, Yeovil, UK) containing 10 % fetal bovine serum (FBS "Gold", PAA, Yeovil, UK) and antibiotics (1 % penicillin/streptomycin, and 1 % fungizone, Invitrogen, Paisley, UK). Cells were maintained at 37°C with constant humidity in a 5 % CO_2-95 % air atmosphere. The growth medium was changed every 2-3 days. For uptake studies, cells were seeded into 6-well plates (seeding density of ca. 5.4 x 10^4 cm^{-2}) and used for experiments post confluence (11 days or 12 days post-seeding for the 'normal' 6-well plates, i.e. non-transwell) to allow formation of an intact monolayer and cellular differentiation. To obtain the TEER measurements cells were seeded into 12-well transwell plates (Greiner, Stonehouse, UK) and used at 21 days post-seeding. All experiments for this study were conducted at passages 24 – 50.

TEER Measurements

An electrode (STX2) interfaced with a volt-ohm meter (EVOM2, WPI, Sarasota, USA), was used to assess TEER of Caco-2 cell monolayers grown on 12-well transwell plates. This was used as a measure of cell monolayer integrity and to detect early effects causing an opening of tight junctions. TEER of the inserts was assessed on day 21 of culture to confirm presence of a confluent monolayer. Inserts with TEER lower than 350 Ω were discarded. The volt-ohm meter was calibrated and the electrode equilibrated in pre-warmed medium before each measurement.

TEER measurements were taken at different time points during experiments to monitor membrane integrity. Briefly, MEM was aspirated from the insert and the Caco-2 cell monolayer was washed with pre-warmed phosphate buffered saline (PBS). MEM cell culture medium ± Fe and/or ascorbic acid was then added to the cells and the first TEER measurement was taken after ca. 10 min equilibration time (referred to as t_0). Further measurements were taken at 1, 2 and 3 hours incubation or just at 1 hour incubation with iron.

The media were then aspirated at appropriate times and cells were washed three times with PBS-EDTA to remove cell membrane adherent iron and fresh un-supplemented MEM was added. The cells were returned to the incubator and a final TEER measurement was taken at 24 hours (t_{24}). Results are expressed as percentage change in TEER ± SD in relation to t_0. The range of t_0TEER values for the experiments was 400-1058 Ohm.cm^2.

Cellular Uptake Studies

Caco-2 cell monolayers were changed to MEM alone (i.e. without FBS or antibiotics) for 16 hours prior to washing with pre-warmed PBS. Cells were then exposed to Fe or vehicle in cell culture medium with each condition being assessed in triplicate. Following a 1 hour incubation period at 37°C the medium was aspirated, the cell monolayer was washed three times with PBS-EDTA to remove cell membrane adherent iron and fresh MEM was added. The cells were then returned to the incubator for an additional 23 hours incubation period to allow for ferritin formation. Cells were then lysed by addition of MPER® (Mammalian Protein Extraction Reagent lysis buffer [Thermo Scientific, Loughborough, UK]) according to the manufacturer's protocol. Briefly, 400 µl of MPER® lysis buffer was added to each well and cells were detached by gently shaking the plate. The lysate was collected, centrifuged for 5 min at 16,000 *xg*, and the supernatant was collected for analysis.

Total protein content of the Caco-2 cells was measured by the NIPA (non-interfering protein assay, Calbiochem [Merck], Nottingham, UK) according to the manufacturer's protocol. Briefly, standards were prepared in the range 0 – 300 µg/ml and analysed in duplicate. Fifty microliters of pre-diluted sample were pipetted in triplicate to the micro-plate and 20 µl of copper solution was added. After thorough mixing, 100 µl of reagent II were added and the plate incubated in the dark for 10 min at room temperature before reading the absorbance at 490 nm.

Ferritin content of Caco-2 cells was determined using the ELISA kit "Spectro Ferritin" (ATI Atlas, Chichester, UK) following the manufacturer's protocol. Briefly, 10 µl of pre-diluted sample were added to solid phase antihuman ferritin micro-wells. After addition of 200 µl conjugated antihuman ferritin, the micro-wells were incubated for 2 hours on a rotator table at 180-200 rpm at room temperature followed by washing the wells three times with deionized water and then the addition of 200 µl substrate solution. Colour was developed after 30 min incubation by addition of 100 µl potassium ferricyanide. Absorbance was read at 490 nm against a calibration range using concentrations between 3 and 2000 ng/ml and corrected for background at 595 nm.

Baseline ferritin was determined in every experiment on cells that received the same treatment as the experimental groups except Fe was not added to the media (i.e. controls). Results are presented corrected for the baseline values and expressed in relation to total cell protein in ng ferritin/mg cell protein. Typically, ferritin concentration for the control cells was that of baseline ferritin levels (ca. 2-9 ng/mg cell protein) confirming the very low iron availability of the control media [16-18].

Statistical Analysis

All results are presented as means ± SD from independent experiments (n=3).

The two-way analysis of variance (ANOVA) procedure was performed using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego California USA) to determine significance ($P<0.05$). The Bonferroni post-test was used to test which means differed.

RESULTS

Typically, isotopically-labelled Fe(II) sulphate (± ascorbic acid) is used at low (≤10 µM) concentrations in iron uptake experiments in cultured cells [5, 13, 19-21]: however, to represent gut luminal levels of iron that correspond to supplemental Fe doses, we calculated that concentrations ca. 200 µM are required. These estimated concentrations are based on a 60 mg supplemental Fe dose undergoing dilution in 0.5 litre gastric juice and a further dilution of 1:10 in the duodenum. This value also accounts to 10mg day of dietary non-haem iron, split mainly between 3 meals, leading to duodenal [Fe] of 10 µM [1] (thus a simple dose of 60mg would lead to 60 mg/(10 mg/3 meals) x 10 µM = 180 µM).

First, therefore, we considered the phase distribution of the iron at these high Fe concentrations in TCM (MEM). Only when ascorbic acid was added to 200 µM Fe(II) sulphate, at a molar excess of 100 fold or more, was the Fe mostly soluble. At lower ascorbic acid levels, the iron was nanoparticulate (in suspension) or precipitated (Figure **1**).

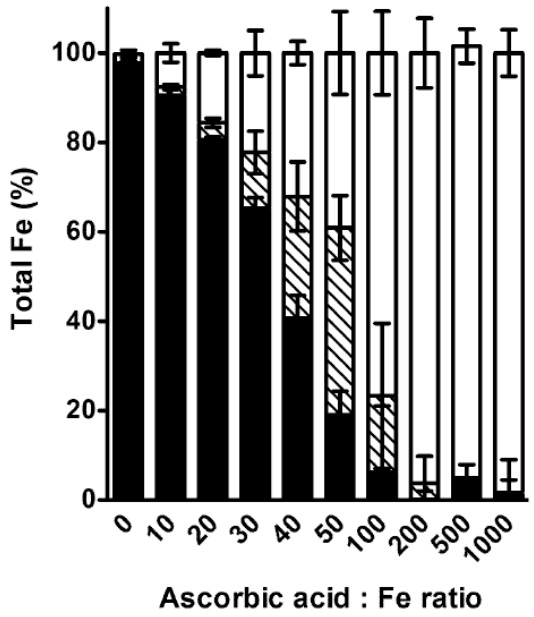

Figure 1: Phase distribution of 200 µM $FeSO_4$ in MEM with increasing concentrations of ascorbic acid. Microparticulate (closed bars) refers to the percentage of iron present in the pellet after centrifugation; nanoparticulate (pattern bars) refers to the percentage of iron that remained in suspension with centrifugation, but did not ultrafilter through a 3 kDa MWCO membrane while the soluble iron (open bars) was ultrafilterable. Values are shown as mean ± SD of 3 independent experiments.

We next considered whether the required supra-physiological levels of ascorbic acid in the medium would disturb the cellular monolayer. Appropriate measures of Fe uptake require an integral confluent monolayer and early signs of disruption of the monolayer can be assessed by investigating TEER (Figure **2**). We showed that there was a disruption of the cell monolayer, indicated by a significant (*P<0.05*) drop in the TEER, when the cells were incubated with ascorbic acid levels of 20 mM and above for 3 hours and then left for up to 24 hours in fresh media with no ascorbic acid (Figure **2A**). When 200 µM Fe(II) sulphate was also added to the media the ascorbic acid effects were exacerbated with marked deceases in TEER (*P<0.001*) for all time points with the exception of 20 mM ascorbic acid at the early time points, although by 24 hours the effect on TEER was similarly dramatic (*P<0.001*) for iron plus 20 mM ascorbic acid (Figure **2B**).

In spite of the findings above, when cells were incubated with 20 mM ascorbic acid with or without Fe for only one hour (arguably a more physiological time point) and the supplemented media was then removed and replenished with fresh MEM for a further 23 hours, ascorbic acid (± Fe) was less detrimental to the

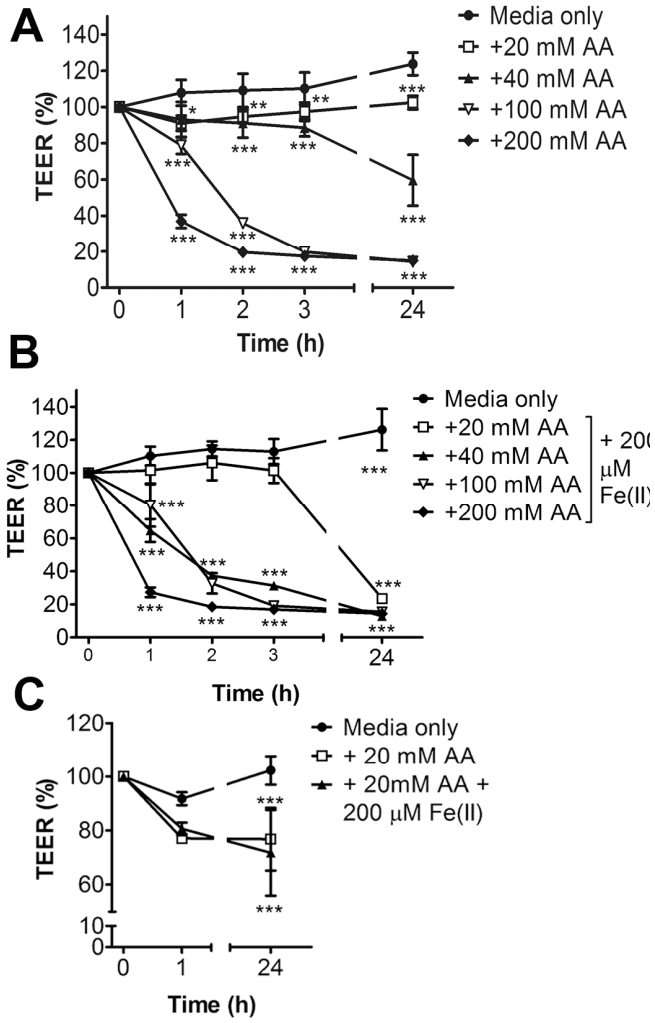

Figure 2: TEER changes at different time points during incubation with: (**A**) MEM supplemented with ascorbic acid (AA) at different levels over 3 hours; (**B**) MEM supplemented with ascorbic acid at different levels plus 200 µM Fe over 3 hours; or (**C**) MEM supplemented with ascorbic acid alone or ascorbic acid plus iron in a fixed molar ratio of 100:1 for just 1 hour ± a further 23 hours in fresh un-supplemented MEM. Values are percentage of the initial measurement and are shown as mean ± SD of independent experiments (n=3). Experimental points are connected with a solid line for ease of read and not because a linear relationship is assumed between time and TEER measurement. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ in relation to 'media only'.

monolayer with only an approximate 20 % decrease in TEER at 24 hours (P<0.001) (Figure **2C**).

Ferritin formation by Caco-2 cells is a useful marker of Fe utilization by the cells, and therefore we assessed if the cells were able to utilize the Fe from Fe(II) sulphate in the conditions chosen (i.e. 1 hour exposure to [Fe] = 200 µM, [ascorbic acid] ≤ 20 mM). As expected, ferritin formation increased as a function of iron solubility (Figure **3**). However, we noted that the addition of 20 mM ascorbic acid to cell culture medium (MEM) induced a fall in pH to ~ 5.7, and when the pH

was re-adjusted to 7.4 Fe again started to precipitate with only 30 % of Fe remaining truly soluble (the remaining being ca. 50% as a true precipitate and ca. 20% as a nano-precipitate in suspension).

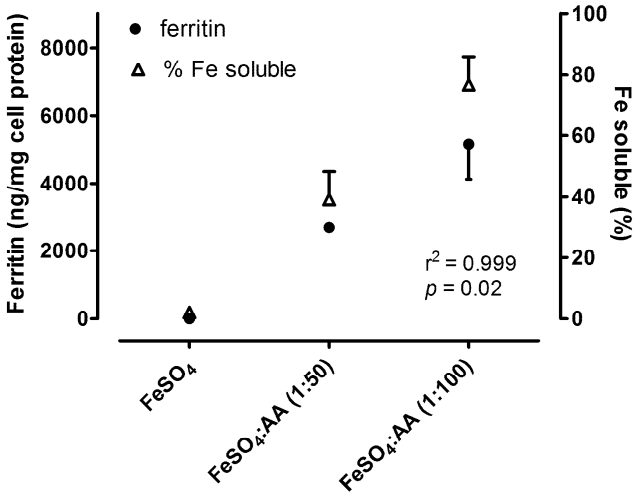

Figure 3: Ferritin content of a Caco-2 cell monolayer after 24 hours following 1 hour incubation with Fe at 200 µM either alone or with two different levels of ascorbic acid (AA) (primary Y-axis). The secondary Y-axis shows the percentage of Fe that remains soluble in the supplemented MEM. Values are shown as mean ± SD of independent experiments (n=3). The Pearson correlation between ferritin and soluble Fe is significant with p=0.02 and r^2=0.999.

Conversely, another therapeutic iron supplement, namely Fe(III) maltol, is soluble at peri-neutral pHs at mM Fe concentrations (for example 8-10 mM). Indeed, with even simple mixing of the two components in cell culture medium only a small proportion of the Fe appeared colloidal/insoluble at a ratio of 5:1 (maltol:iron) (Figure **4A**). But, in contrast to the Fe plus ascorbic acid system, this chelate showed no marked influence on the monolayer TEER at any of the time points investigated (Figure **5A**, **B**) while the iron was still efficiently utilized by the cells at pH 7.4 (Figure **4B**).

Taken together these data suggest that Fe(III) maltol is a suitable vehicle for delivery of soluble Fe to Caco-2 cells at these supplemental Fe levels.

DISCUSSION

The Caco-2 cell model is considered a useful screening technique for Fe uptake and bioavailability studies [5, 19, 22-28]. When working with low (≤10) µM concentrations, which are representative of dietary and fortificant iron levels, Fe(II) sulphate or Fe(II) sulphate with ascorbic acid are commonly used as reference or positive control compounds [29, 30]. However, as shown in this study at supplemental levels, Fe(II) sulphate does not remain soluble in typical cell culture

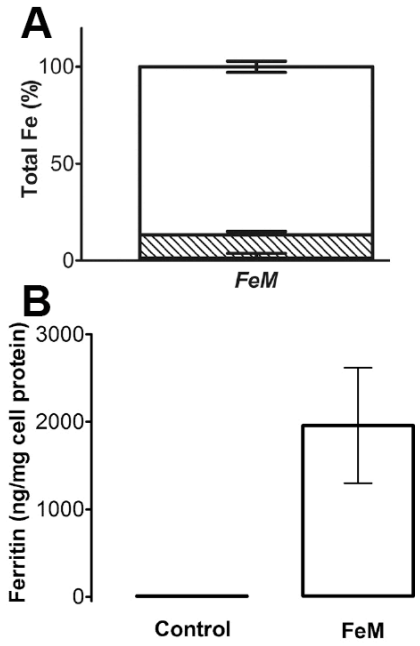

Figure 4: Fe(III) maltol utilization by Caco-2 cells. (**A**) Phase distribution of 200 µM Fe(III) maltol at a ratio of 5:1 (maltol:iron) at pH 7.4 in uptake medium. Microparticulate (closed bars) refers to the percentage of iron present in the pellet after centrifugation; nanoparticulate (pattern bars) refers to the percentage of iron that remained in suspension with centrifugation, but did not ultrafilter through a 3 kDa MWCO membrane while the soluble iron (open bars) was ultrafilterable. Values are shown as mean ± SD of independent experiments (n=4). (**B**) Ferritin content of a Caco-2 cell monolayer after 24 hours following 1 hour incubation with BSS supplemented with 200 µM Fe(III) maltol (FeM; 1:5 ratio) and 23 hours in fresh un-supplemented MEM. Control values represent baseline ferritin levels in cells incubated in the same conditions but using un-supplemented BSS for the first hour. Values are shown as mean ± SD of independent experiments (n=3).

media and requires a vast excess of ascorbic acid to achieve full (or significant) solubility (e.g. ratio 1:100 Fe:ascorbic acid). The impact of such high ascorbic acid concentrations (≥ 20 mM) on cellular iron utilization and also on the cellular monolayer integrity was therefore assessed further. It is noteworthy that in many cell iron studies, iron solubility in the TCM is not assessed correctly and 'soluble' iron is quantified as iron that remains in suspension after centrifugation and, as evidenced in this study, this also includes nanoparticulate iron that is not truly soluble (Figure **1**).

We used ferritin formation as a measure of Fe utilization by the cells, which is a credible proxy for Fe uptake as reported, for example, by Glahn and colleagues [29]. This has the advantage of discriminating intracellular and utilisable iron (thus forming ferritin) from simple cell uptake of iron (which includes all fractions of membrane-adherent and intracellular iron). We were able to demonstrate, as

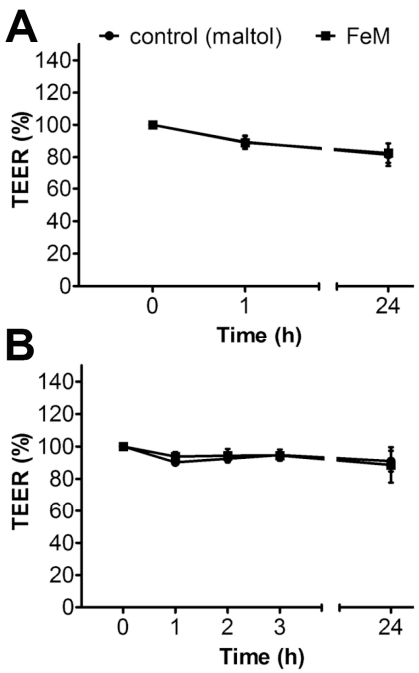

Figure 5: TEER changes to a Caco-2 cell monolayer at different time points during incubation with uptake medium supplemented with 200 µM Fe(III) + maltol (FeM; 1:5 molar ratio). Incubations were for: (**A**) 1 hour (1 h) with Fe plus a further 23 hours in un-supplemented MEM (24 h); or (**B**) 3 hours (measurements at 1-3 h) with Fe plus a further 21 hours in fresh un-supplemented MEM (24 h). Values are percentage of the initial measurement and are shown as mean ± SD of independent experiments (n=3). Experimental points are connected with a solid line for ease of read and not because a linear relationship is assumed between time and TEER measurement.

expected, that ferritin formation increased proportionally with increasing iron solubility: indicative of iron uptake by the soluble Fe(II) transporter (DMT-1) [31] matching Fe(II) in solution.

In order to perform reliable studies to investigate iron uptake by the Caco-2 cells, a confluent and integral cell monolayer, that resembles the epithelial barrier *in vivo*, is required. Trans-epithelial electrical resistance (TEER) measurements are a validated method to assess the integrity of the monolayer [14, 32] and here they were used as a tool to detect early signs of changes to the cell monolayer derived from the experimental conditions. According to our TEER measurements, the truly soluble Fe(II) and ascorbic acid mixture (ratio 1:100) caused the cell's tight junctions to open, as evidenced by a drop in TEER, when incubated for 3 hours and this effect persisted up to 24 hours later even after removing the iron and ascorbic acid from the media. This large detrimental effect was not observed when we used an arguably more physiological incubation period (1 hour) representative of the short transit period for iron in the duodenum/upper jejunum. Nevertheless, a significant drop in pH of the cell culture medium was unavoidable when working with the high concentrations of ascorbic acid that were required to maintain Fe(II) in solution and, adjusting the pH to peri-neutral values, caused iron precipitation. Raising ascorbic acid levels further to prevent Fe(II) precipitation at peri-neutral pHs will be of no effect because, as noted above, the Caco-2 cells are not able to maintain an integral monolayer when incubated with Fe(II) in the presence of ascorbic acid concentrations above 20 mM even for as little as 1 hour (Figure **2b**). Maintaining the pH of the cell culture medium for Caco-2 cells is important when undertaking iron uptake/utilization studies since small changes in pH will greatly affect uptake *via* the soluble iron transporter (DMT1) [8, 33].

We therefore investigated the usefulness of a Fe(III) iron chelate, namely Fe(III) maltol (here with a ratio of Fe:maltol at 1:5), as a suitable alternative in these conditions. Fe(III) maltol is a soluble iron compound that has been shown to be efficacious at correcting chronic iron deficiency anaemia in patients intolerant to Fe(II) compounds [34] which suggests that it is less aggressive (toxic) than Fe(II) salts towards the epithelial layer of gut cells. Prior work has also shown that Fe(III) maltol uses the normal iron regulatory pathways, specifically the divalent metal transporter DMT-1 for absorption into gut cells [35]. Fe(III) maltol showed a reproducible phase distribution profile with about 90 % of the iron being soluble in the TCM. The pH of Fe(III) maltol in the TCM was stable at 7.4. In contrast to the soluble Fe(II) plus ascorbic acid system, neither maltol nor the Fe(III) maltol complex caused any marked changes in TEER over the studied time periods, suggesting this compound does not interfere with monolayer integrity, which supports its safety profile observed clinically [34] and in animal studies [36].

Furthermore there was significant and reproducible iron utilization by the Caco-2 cells, to produce ferritin, when cells were incubated with Fe(III) maltol showing uptake and metabolism of the chelate. Therefore, we suggest that Fe(III) maltol preparations are a more reliable and appropriate soluble Fe control than Fe(II) based systems, when working at supplemental Fe levels in Caco-2 cells.

In this study we describe an appropriate experimental setup using the Caco-2 cell model with Fe concentrations typical of those found in the intestinal lumen after an oral iron therapeutic dose. The Caco-2 model with the experimental conditions developed

here, such as the reference soluble Fe material, is a useful tool to screen novel supplemental iron compounds and advance the development of better oral Fe for the treatment of iron deficiency anaemia.

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STATEMENT OF AUTHORS' CONTRIBUTIONS TO MANUSCRIPT

B.I.M., J.J.P. and D.I.A.P. designed the research; B.I.M., E.R., D.I.A.P. conducted research; S.F.A.B., provided essential materials; B.I.M. and D.I.A.P. analysed data; B.I.M., J.J.P. and D.I.A.P. wrote paper; D.I.A.P had primary responsibility for final content. All authors read and approved the final manuscript.

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