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Co-administration of curcumin forms with supplemental iron: a study of effects on iron absorption and intestinal cellular health

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Iron deficiency (ID) and iron deficiency anaemia (IDA) are significant global public health issues, with their main line of treatment being oral iron supplementation that often leads to gastrointestinal (GI) adverse effects and early discontinuation. Strategies to improve the efficiency of oral iron supplements and to ameliorate their associated GI effects could help resolve ID and IDA. Curcumin, a potent antioxidant and anti-inflammatory, iron chelating compound from turmeric may mitigate some of these issues. This study aimed to mechanistically assess the interaction of iron (20 and 100 μM) with two different forms of curcumin – native, unformulated curcumin *versus* a formulated curcumin (HydroCurc®) at 5, 10 and 20 μM , and the consequent effects on iron uptake and intestinal cellular health. Presence of formulated curcumin incremented ferritin levels by 160.5% *versus* free iron treatment alone ($p < 0.0001$) in intestinal Caco-2 cells. A greater ferric iron reducing power by the formulated curcumin was observed ($p < 0.001$). Formulated curcumin also protected against iron-induced permeability of the intestinal barrier ($p < 0.05$). Our data points to formulated curcumin's ability to limit oxidation of divalent iron as a possible mechanism to explain the greater iron uptake by intestinal cells. This study characterises for the first time the distinct interaction behaviour of curcumin forms with iron and the potential consequences on iron uptake and intestinal health, providing novel insights that may help optimise therapeutic approaches to tackle ID and IDA.

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Introduction

Iron deficiency (ID) is a worldwide public health nutritional disorder that accounts for more than 60% of anaemia cases.^{1,2} Anaemia, defined as a low blood haemoglobin concentration ($<12 \text{ g dL}^{-1}$ for women and $<13 \text{ g dL}^{-1}$ for men),³ affects populations in low, middle- and high-income countries and is attributed to significant adverse health, social and economic consequences.⁴ Indeed, iron deficiency anaemia (IDA) was found to be one of the five leading causes influencing a great number of years lived with disability (years of life lived in less-than-ideal health⁵) in a study considering 328 diseases and injuries in 195 countries between 1990 and 2016.⁶ In 2011, 29% (496 million) of non-pregnant women and 38% (32.4 million) of pregnant

women aged 15–49 years were anaemic. This has driven the World Health Organisation (WHO) goal of a 50% reduction of anaemia in women of reproductive age by 2025;⁷ however, recent analysis of pooled global data suggests progress on this has been insufficient. Consequently, discussions are being held to extend the WHO target on anaemia to 2030.⁸

The current gold standard treatment of ID and IDA is *via* supplementation using ferrous salts, primarily ferrous sulphate.^{9,10} Often, oral iron is a cost-effective approach, but it also causes gastrointestinal (GI) side effects that affect patient adherence to treatment, leading to unresolved ID and IDA.^{9,11} The GI symptomatology is caused by the retention of excessive free labile iron in the gut lumen due to limited fractional iron absorption (proportion of iron absorbed) from oral ferrous sulphate (reported to be as low as 9–22%).¹² This leads to large unabsorbed bolus doses of reactive free iron in the intestine that have been associated with endoscopically identifiable injury in the intestinal mucosa.¹³ Excess unbound ferrous iron increases the production of reactive oxygen species (ROS) *via* Haber–Weiss and Fenton reactions.^{14–19} Iron-induced ROS is linked to a disruption of the integrity of the intestinal membrane tight junctions, a key element for intestinal epithelial

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integrity and selective permeability.^{17,18,20} With continued oral iron therapy, increased levels of accumulated unbound iron occur and consequently increasing levels of ROS. This leads to oxidative stress-induced lipid peroxidation of cellular membranes and mitochondrial and endoplasmic reticulum activation of cell death pathways.^{15–18} The destruction of the epithelial cell layer eliminates the mechanical intestinal barrier and increases permeability, known as leaky gut.²¹ Iron supplementation associated GI issues have also been reported to alter the balance of the gut microbiota in favour of pathogenic bacteria.^{17,22–25} These events manifest clinically in 30–70% of patients^{9,26} as the undesirable GI adverse effects (*i.e.*, nausea, diarrhoea, stomach ache and constipation)⁹ that ultimately lead to non-compliance in 60% of cases.^{26–28} Hence, novel approaches designed to enhance tolerance to iron supplementation are needed to help combat ID and IDA.

Co-administration of oral iron with compounds aimed at countering iron-mediated GI damage may thus represent an innovative approach to oral iron therapy. Research in this area has suggested that co-administration with antioxidant-rich supplements has the potential to reverse the iron supplement-induced depletion of faecal antioxidant capacity.²⁹ Curcumin is a naturally occurring plant polyphenol that has demonstrated potent antioxidant power and anti-inflammatory activity.^{30–32} Furthermore, the role of curcumin in the protection of gut health was evidenced *via* restoration of the intestinal barrier and modulation of the microbiota.^{33–36} The results of the FeROUTINE trial, the first large-scale human study on the combined effect of iron and curcumin, reported a very low incidence of iron-related GI symptoms following a 6-week supplementation period.³⁷ This trial also found a reduction of markers of systemic oxidative stress and inflammation over time.³⁸ Notably, since curcumin can form iron chelation complexes,^{39–41} it is critical to assess the potential impact that co-administration may have on physiological iron levels. In this regard, the FeROUTINE trial reported that the curcumin in the form of HydroCurc® did not affect the absorption of iron.^{37,42} HydroCurc® is a commercially available, formulated form of curcumin entrapped in a delivery system that has been shown to enhance the bioavailability of curcuminoids in humans.⁴³ In contrast, previous research demonstrated the ability of curcumin to deplete iron and induce iron deficiency in cellular and animal models, pointing to this polyphenol as an effective agent for treating iron excess diseases.^{44–46} However, it is pertinent to note that these studies were conducted with native, free, unformulated curcumin. Further research would help clarify whether native and formulated forms of curcumin may distinctly influence iron uptake and iron homeostasis. Equally, despite the relative absence of GI symptomatology reported in the FeROUTINE clinical trial, there is lack of quantitative data on the potential of forms of curcumin to protect against oral iron-induced GI damage. Thus, the present research aimed to mechanistically assess the effect of combined iron and curcumin forms (native unformulated curcumin and formulated curcumin – HydroCurc®) on iron uptake and intestinal cellular health utilising the well-established Caco-2 human intestinal cell-based *in vitro* model.

Materials and methods

Unless otherwise stated, all chemicals were analytical grade or cell culture grade where applicable. Reagents were always prepared using ultrapure water (water purified through a 0.22 µm membrane filter with a resistivity of 18.2 MΩ).

Caco-2 cells were obtained from European Collection of Cell Cultures (Catalogue No. 09042001, ECACC, Salisbury, UK). The enzyme-linked immunoassay (ELISA) test kits for ferritin determination were from Eagle Biosciences, Inc. (Nashua, USA). Formulated curcumin (HydroCurc®, Gencor Pacific Limited, Hong Kong) consisting of 85% total curcuminoids entrapped in a proprietary delivery system (LipiSpere®, Pharmako Biotechnologies Pty Ltd, New South Wales, Australia) was kindly provided by Pharmako biotechnologies Pty Ltd (New South Wales, Australia). Standard curcumin (98% curcumin, (10286890)), ferrous sulphate heptahydrate (FeSO₄·7H₂O) (11472178), ferric chloride anhydrous (012357.22), methanol (HPLC grade, ≥98%), 10× Gibco™ Dulbecco's – Phosphate Buffered Saline (DPBS) (14190-044), Corning™ Dimethyl Sulfoxide (DMSO) (15303671), Gibco™ Dulbecco's Modified Eagle's Medium (DMEM) (11584456), Gibco™ Minimum Essential Medium (MEM) (51200-0.46), Gibco™ Foetal Bovine Serum (FBS) (11550356), Gibco™ Antibiotic-Antimycotic (100×) (11570486), Gibco™ L-Glutamine (200 mM) (11500626), Gibco™ HBSS with calcium and magnesium, no phenol red (11550456), Gibco™ HEPES (1 M) (11560496), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (L11939.06), the protease inhibitor cocktail (78429), Pierce BCA kit (23227), the Costar Corning™ (ref. 3599) cell culture-treated, flat bottom, 96-well plates (10792552), the Nunclon delta surface 6-well plates, the Corning™ 3412 Transwell® membrane inserts (10301031), and the cell scrapers (08-100-241) were from Fisher Scientific Ltd (Loughborough, England, UK). The black-walled, clear bottom, Corning™ Ref. 3603 cell culture-treated, the 96-well plates (10530753), and the 25-gauge needles (BD414) were purchased from Appleton Woods Ltd (Birmingham, England, UK). Sodium hydroxide (30620), 2',7'-Dichlorofluorescein diacetate or DCFDA, 2,2'-Azobis(2-methylpropionamide) dihydrochloride or ABAP (440914-25G), 2,4,6-Tris(2-pyridyl)-s-triazine or TPTZ (T1253) and 2-(*N*-Morpholino) ethanesulfonic acid (MES) hydrate (M8250), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid or Trolox (238813-5G) and Lucifer yellow (L0144) were from Merck Life Science UK Limited (Dorset, England, UK).

Test sample preparation

Stock solutions of native curcumin and HydroCurc® were prepared in methanol and protected from light with aluminium foil. Ferrous sulphate heptahydrate (20% elemental iron) was dissolved in 0.1 M HCl and ferric chloride (34.42% elemental iron) in ultrapure water. These stocks were used to prepare working solutions at varying target concentrations for each specific assay in either ultrapure water or phenol red- and FBS-free MEM. Ferrous iron (Fe²⁺) was used the default form of iron assayed in all experiments. Ferric iron (Fe³⁺) was used in



selected experiments as indicated below. Where titration of media was required, 0.1 and 1 M HCl and 50 mM and 1 M NaOH were used to achieve a final pH of 5.8 (representative of duodenal pH) and sterilised by passing it through a 0.22 μm sterile filter. The titrated, sterile-filtered MEM was then buffered by adding 0.5 M sterile-filtered 2-(*N*-Morpholino) ethanesulfonic acid (MES) to achieve a final concentration of 10 mM MES.

Ferric iron reducing antioxidant power (FRAP) assay

The ability of native curcumin and HydroCurc® to reduce iron from ferric to ferrous oxidation state was assessed following the modified protocol by Zupancic *et al.*, 2014.⁴⁷ The FRAP reagent solution was prepared by mixing 10 mM tripyridyl triazine (TPTZ), 20 mM ferric chloride, and 300 mM acetate buffer (pH 3.6) at a ratio of 1:1:10 (acetate buffer:TPTZ:ferric chloride). 30 μL of test compounds were allowed to react with 900 μL of FRAP reagent for 30 minutes at 25 °C, and absorbance was read at 593 nm. FRAP reagent was used as blank. A standard curve with Trolox was prepared since Trolox is a recommended baseline antioxidant reference point for multiple antioxidant assays.^{48,49} Absorbance values of test compounds were interpolated, and results were expressed as Trolox Equivalents (TE).

Iron chelation activity

The fluorescence of curcumin reduces upon metal chelation. Therefore, the iron chelating behaviour of curcumin forms can be monitored by tracking its fluorescence as it is combined with iron.^{31,50} Native curcumin or HydroCurc® were combined with ferric or ferrous iron in ultrapure water to a final concentration of 20 μM for the curcumin forms and 100 μM for the iron. The controls were 20 μM native curcumin or HydroCurc®, iron-free solutions. The preparations were covered in foil and allowed to react for 30 minutes at room temperature with gentle agitation (100 rpm). 200 μL from each solution were dispensed in a black-walled, clear bottom 96 well plate. Fluorescence was measured at 485 excitation and 520 emission.

Caco-2 cell culture

Caco-2 cells (passage 20–56) were cultivated in DMEM – Glutamax®, pH 7.4, supplemented with 10% FBS, 10% L-glutamine and 1% antibiotic/antimycotic in a 5% CO₂, constant humidity environment at 37 °C. Media was replenished every other day, and cells were trypsinised at 70–90% confluence and seeded onto the relevant plate for experimentation, as previously described.^{51,52}

Iron uptake experiment and determination of ferritin in Caco-2 cells

Caco-2 cells were seeded (4 500 cells per cm²) onto 6-well plates and cultured for a minimum of 12 days to allow cells to fully differentiate into mature enterocytes and form a monolayer.^{53,54} Media was replenished every 2–3 days. The day before the experiment, cells were washed with DPBS and incu-

bated in serum-free MEM for 24 h. Stock solutions of iron and curcumin forms were prepared fresh on the day of the experiment, as described above. Final test media was prepared by titrating MEM with 0.1 and 1 M HCl and 50 mM and 1 M NaOH to achieve a final pH of 5.8 (representative of duodenal pH⁵⁵). Titrated MEM was sterile-filtered by using a 0.22 μm filter unit and then buffered with 2-(*N*-Morpholino) ethanesulfonic acid (MES, 10 mM). Varying volumes of stock solutions were pipetted into the titrated, MES-buffered MEM to achieve the target final concentrations detailed next. Test conditions were 100 μM ferrous iron alone and combined with either native curcumin or HydroCurc® (5 and 10 μM). Buffered MEM was used as control. Caco-2 cells were incubated with test media for 2.5 hours (representative of duodenal transit time)⁵⁶ at 37 °C, 15–30 rpm, protected from light. Following removal of conditions, cells were incubated for a further 22.5 hours (24 h in total) in MES-buffered MEM (37 °C, 5% CO₂) to allow cells to store uptaken iron as ferritin. Caco-2 cells were lysed at 4 °C as described previously⁵¹ with 350 μL per well of ice-cold lysis buffer (50 mM NaOH supplemented with 1 $\mu\text{g mL}^{-1}$ protease inhibitor cocktail) for 40 minutes with gentle shaking. Lysates were scrapped, passed through a 25-gauge needle to reduce viscosity, aliquoted and kept at –20 °C for further analyses. Ferritin cellular content was measured using an enzyme-linked immunoassay (ELISA) test kit (Eagle Biosciences, Inc., Nashua, USA) following manufacturer instructions with some modifications. Briefly, 20 μL of the sample were loaded in duplicate on the pre-coated wells and incubated for 60 minutes at 200 rpm with the mouse anti-ferritin antibody-horseradish peroxidase (HRP) conjugate. Subsequent steps were carried out as described in the protocol. The protein content of the Caco-2 cells was determined using the Pierce BCA kit following the manufacturer's protocol. Ferritin concentration was standardised against total protein concentration and expressed as ng ferritin per mg protein. Ferritin was used as a surrogate marker of iron entry and storage in the cells. Ferritin was previously shown to increase proportionally in response to soluble iron and a reliable proxy for iron uptake in Caco-2 *in vitro* model.^{54,57,58}

Cell viability assay

Caco-2 cells were seeded (30 000–45 000 cells per cm²) on clear 96-well plates and cultured for 6–7 days. Test samples were prepared fresh on the day in phenol red-free MEM. Test conditions were ferrous iron (20 and 100 μM) alone and combined with either native curcumin or HydroCurc® (5, 10 and 20 μM). Control was phenol red-free MEM. Following 24h incubation (37 °C, 5% CO₂) of the Caco-2 cells in these preparations, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (5 mg mL⁻¹ DPBS) was added to all wells and plates were further incubated for 4 hours. Wells were aspirated, MTT formazan product solubilised with 100 μL of DMSO for 15 minutes at 37 °C, 125 rpm, and absorbance read at 570 nm. Cell viability was calculated and expressed as a percentage of the control.



Cellular ROS production

Caco-2 cells were seeded in black-walled, clear bottom 96-well plates and the assay was performed on day 6 or 7 after plating. The experimental protocol was based on the method described by Chen *et al.*, 2015 and Kellett *et al.*, 2018,^{59,60} which is a modified version of the original assay developed by Wolfe *et al.*, 2007 and Hu *et al.*, 2013.^{61,62} Ferrous and ferric iron (20 and 100 μM) alone and combined with either native curcumin or HydroCurc® (5 and 20 μM), and curcumin forms (native curcumin and HydroCurc®, 5 and 20 μM) were prepared in MEM. 200 μL of these solutions were added to test wells. Control and blank wells contained only MEM. Following 1 h incubation (37 °C, 5% CO_2), wells were aspirated, washed and treated with 200 μL of 25 μM DCFH-DA for 30 minutes (37 °C, 5% CO_2). Subsequently, wells were washed and 100 μL of the pro-oxidant ABAP (2,2'-Azobis(2-methylpropionamide) dihydrochloride) were added to all wells except blank, where MEM was used instead. Fluorescence was read at 520 nm emission and 485 nm excitation every 5 minutes for 1 hour. ROS production was expressed as a percentage of control.

Permeability of the Caco-2 monolayers

A permeable insert based transwell® system was used as an *in vitro* model of the intestinal epithelial barrier to study the absorption of drugs and nutrients as well as to assess potential toxic effects on this biological barrier.^{63–67} Caco-2 cells have been shown to fully differentiate and polarise (develop brush borders at the apical surface, express tight junctions as well as small intestinal microvillus enzymes and transporters) between day 12–21 post seeding on permeable supports.^{63,67,68} This model has been previously used in research investigating the impact of iron on intestinal permeability.^{20,54} Following trypsinisation, the Caco-2 cells were seeded onto polycarbonate permeable membrane inserts (0.4 μm pore size, 24.5 mm outer diameter, tissue culture treated 4.7 cm^2 culture area) and cultured for 21 days. Throughout this period, the transepithelial electrical resistance was routinely measured as a quality control to follow the monolayer development for each insert. The cells were treated with 20 μM ferrous iron and 5 μM HydroCurc®, prepared in titrated and buffered (pH 5.8, 10 mM MES), FBS and phenol red-free MEM, for 24 hours at 37 °C and 5% CO_2 . Following removal of treatments and wash with HBSS, the permeability of lucifer yellow across the monolayer was assessed. Lucifer yellow (LY) is a low molecular weight, hydrophilic compound that passively permeates across the Caco-2 monolayer *via* the paracellular route, *i.e.* through the tight junctions and can be used as a probe to indicate the leakiness of the intestinal barrier.^{67,69} Lucifer yellow solution (100 $\mu\text{g mL}^{-1}$) was prepared in 10 mM HEPES-buffered HBSS Ca^{++} Mg^{++} and 1.5 mL were apically added to the inserts, while 2.5 mL of buffered HBSS Ca^{++} Mg^{++} were added to the basolateral compartment. Following 2.5-hour incubation at 37 °C with gentle agitation (15 rpm), aliquots from each apical and basolateral compartment were loaded onto a black 96-well plate in duplicate. For the standard curve, serial dilutions of 100 $\mu\text{g mL}^{-1}$ LY solution

were prepared. Fluorescence was read at excitation 485 and emission 520. The concentration of LY in each compartment was calculated by interpolation in the standard curve, and the percentage of flux of LY was calculated using the eqn (1) below:

$$\% \text{ Flux} = 100 \times [(F_{\text{I}_b} \times V_{\text{b}})/(F_{\text{I}_a} \times V_{\text{a}})] \quad (1)$$

where F_{I_b} is the fluorescence intensity detected in the basolateral compartment, V_{b} is the volume in the basolateral side, F_{I_a} is the apical fluorescence and V_{a} is the volume in the apical compartment.

Statistical analysis

Data are presented as mean \pm standard deviation of replicates. Ferritin data was analysed by one-way Analysis of Variance (ANOVA) and Dunnett's multiple comparison test. Ferric iron reducing power was analysed by independent, two-tailed *t*-test. The statistical analysis of the chelation activity was conducted by two-way ANOVA followed by *post-hoc* Tukey's multiple comparison test. Cellular viability and ROS production were analysed with one-way ANOVA and corrected with Šidák's multiple comparisons test. Statistical analysis of the permeability of the monolayers was one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparison. All statistical analyses were performed on PRISM software package (Version 10, GraphPad Software Inc., San Diego, USA).

Results

Assessment of iron uptake and storage in the presence of native and formulated curcumin

Statistical analysis indicated there were significant differences between treatments ($F(9,44) = 36.98$, $p < 0.0001$, Fig. 1). Co-treatment with 100 μM ferrous iron and 5 or 10 μM HydroCurc® significantly augmented cellular ferritin concentration by $160.5\% \pm 21.51$ ($p < 0.0001$) and $134\% \pm 37.26$ ($p = 0.0220$) respectively compared to cells treated only with the iron (Fig. 1). 5 μM native curcumin led to a significant increment in Caco-2 ferritin when co-administered with 100 μM ferrous iron ($131.1\% \pm 14.24$, $p = 0.0427$, Fig. 1). Ferritin concentration in cells treated with 10 μM native curcumin plus 100 μM Fe^{2+} was not significantly different from the 100 μM Fe^{2+} condition ($p = 0.1646$, Fig. 1).

Analysis of ferric iron reducing power of curcumin forms

The ferric iron reducing activity of 10 μM HydroCurc® was significantly higher than 10 μM native curcumin ($62.76 \text{ TE} \pm 2.17$ vs. $29.79 \text{ TE} \pm 1.28$, $t(3) = 18.99$, $p = 0.0003$) (Fig. 2).

Evaluation of chelation activity of forms of curcumin

The iron chelation activity of native and formulated curcumin was higher when combined with ferric iron vs. ferrous iron ($F(1, 8) = 311.0$, $p < 0.0001$). Chelation of ferrous and ferric iron by native curcumin was significantly different ($38.33\% \pm 6.88$ vs. $98.20\% \pm 6.68$, $p < 0.0001$). Similarly, chelation by formulated curcumin was significantly lower with ferrous iron



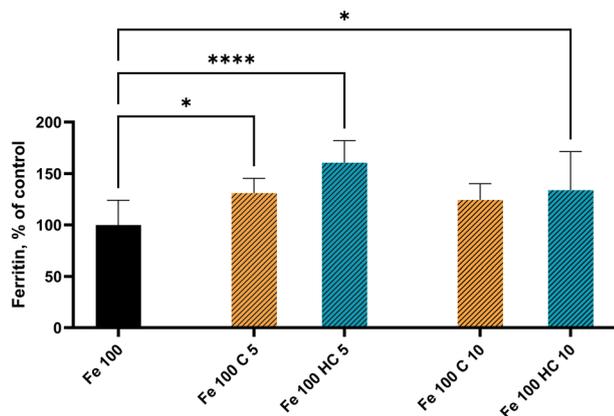


Fig. 1 Effect of 5 and 10 μM curcumin forms (native curcumin (C) and HydroCurc® (HC)) on ferritin concentration in Caco-2 cells treated with 100 μM ferrous iron (Fe). Results are presented as percentage of control (100 μM ferrous iron (Fe 100)) and expressed as mean \pm standard deviation of 6 replicates. Statistical analysis was one-way ANOVA and Dunnett's multiple comparisons test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P < 0.0001$. Combined 5 and 10 μM HydroCurc® enhanced uptake of 100 μM ferrous iron ($p > 0.0001$ and $p = 0.0220$, respectively). Only 5 μM native curcumin resulted in enhanced iron uptake by the cells ($p = 0.0427$), as 10 μM native curcumin did not have a significant effect.

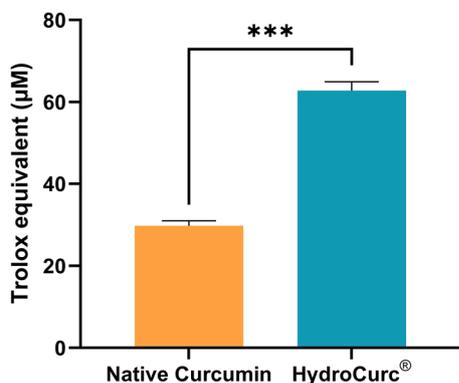


Fig. 2 Ferric iron reducing activity of 10 μM native curcumin and 10 μM HydroCurc®, expressed as Trolox Equivalents. Data are presented as mean \pm standard deviation of 3 replicates and were analysed by independent, two-tailed *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P < 0.0001$. 10 μM formulated curcumin demonstrated a greater ability to reduce ferric ions to their ferrous state compared to 10 μM native curcumin ($p = 0.0003$).

than with ferric iron ($22.86\% \pm 7.11$ vs. $91.48\% \pm 4.10$, $p < 0.0001$) (Fig. 3).

Viability of Caco-2 cells

The assayed treatments led to significant differences in Caco-2 viability following 24h incubation ($F(14,73) = 16.69$, $p < 0.0001$, Fig. 4). The formulated curcumin in combination with either 20 or 100 μM ferrous iron did not cause significant changes in Caco-2 cell viability vs control. The viability of the cells treated with 10 and 20 μM native curcumin in combination with

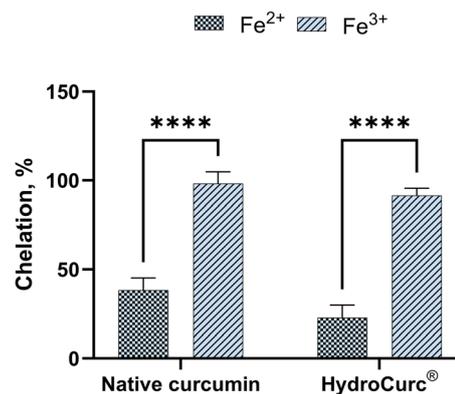


Fig. 3 Chelation activity of forms of curcumin (20 μM native curcumin or formulated curcumin, *i.e.*, HydroCurc®) combined with ferric and ferrous iron (100 μM). Data are presented as mean \pm standard deviation of 3 replicates and analysed by two-way ANOVA followed by *post-hoc* Tukey's multiple comparison test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P < 0.0001$. Both native and formulated curcumin demonstrated a significantly higher chelation activity with ferric iron than with ferrous iron ($p < 0.0001$).

100 μM iron lowered to $44.23\% \pm 4.73$ and $52.04\% \pm 6.35$, respectively ($p < 0.0001$ vs. control). The mean difference of these values versus 100 μM iron was 63.55% (95% CI: 40.32 to 86.78, $p < 0.0001$) and 55.74% (95% CI: 32.51 to 78.96, $p < 0.0001$). In addition, the viability of the Caco-2 cells co-treated with 20 μM native curcumin and 20 μM iron ($60.35\% \pm 4.62$) was lower than control ($p < 0.0001$). Distinctly, 10 μM native curcumin augmented cellular viability when combined with 20 μM iron to $113.7\% \pm 21.54$ ($p = 0.0015$ versus 20 μM iron) (Fig. 4).

Reactive oxygen species (ROS) levels in Caco-2 cells

Co-treating Caco-2 cells with curcumin forms and iron (20 μM or 100 μM) generated distinct levels of ROS in the Caco-2 cells (20 μM iron: $F(7,24) = 19.88$, $p < 0.0001$, Fig. 5A). 100 μM iron: $F(7,24) = 65.27$, $p < 0.0001$, Fig. 5B). The treatments involving 20 μM ferrous iron (Fe^{3+}) in combination with the formulated curcumin (5 and 20 μM) increased ROS production by 1.49- and 1.54-fold, respectively, compared to the analogue native curcumin treatments ($p < 0.0001$, Fig. 5A). Similarly, when the cells were incubated with ferric iron (Fe^{3+}) treatments, formulated curcumin increased ROS by 1.22- and 1.2-fold compared to native curcumin ferric combinations (5 μM curcumin: $p = 0.0138$, and 20 μM curcumin: $p = 0.0377$, Fig. 5A). Finally, combinations of 100 μM ferrous iron with 5 and 20 μM formulated curcumin increased Caco-2 ROS production by 1.38- and 1.42-fold compared to native curcumin ($p < 0.0001$, Fig. 5B). Likewise, the analogue ferric iron combinations of formulated curcumin also led to significant increases of cellular ROS (1.26 and 1.22-fold, respectively, $p < 0.0001$, Fig. 5B).

Assessment of the permeability of Caco-2 monolayers

Incubating the Caco-2 cells with 20 μM ferrous iron alone and combined with 20 μM native or formulated curcumin induced



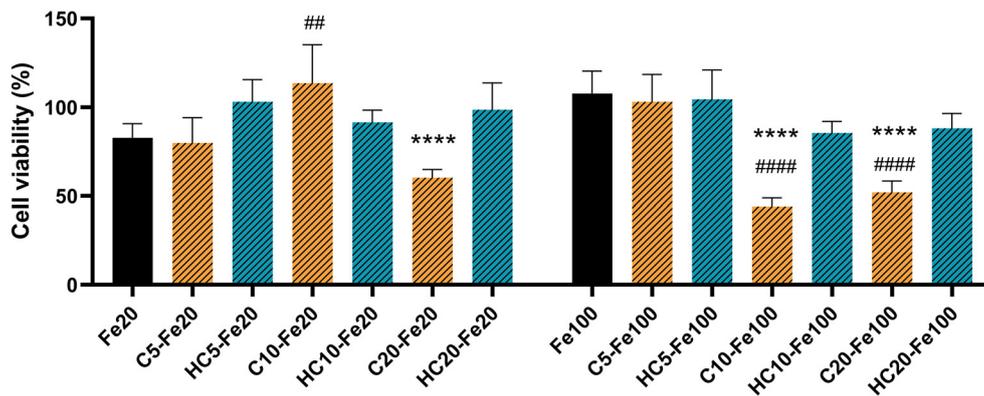


Fig. 4 Caco-2 cell viability expressed as percentage of control (MEM) upon 24 h treatment with ferrous 20 and 100 μM iron (Fe20 and Fe100) alone and combined with 5, 10 and 20 μM either native curcumin (C5, C10 and C20) or formulated curcumin (HC 5, HC10 and HC20). Data are presented as mean \pm standard deviation of 6 replicates and analysed by two-way ANOVA followed by *post-hoc* Šidák's multiple comparisons test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P < 0.0001$. (*) Symbol indicates significant difference versus control (MEM). (#) symbol indicates significant difference versus the relevant concentration of iron. 10 and 20 μM native curcumin in combination with 100 μM iron led to lower viability than control and 100 μM iron ($p < 0.0001$). 20 μM native curcumin in combination with 20 μM iron resulted in toxicity vs control ($p < 0.0001$). 10 μM native curcumin in combination with 20 μM iron led to Caco-2 proliferation vs Fe20 ($p = 0.0015$).

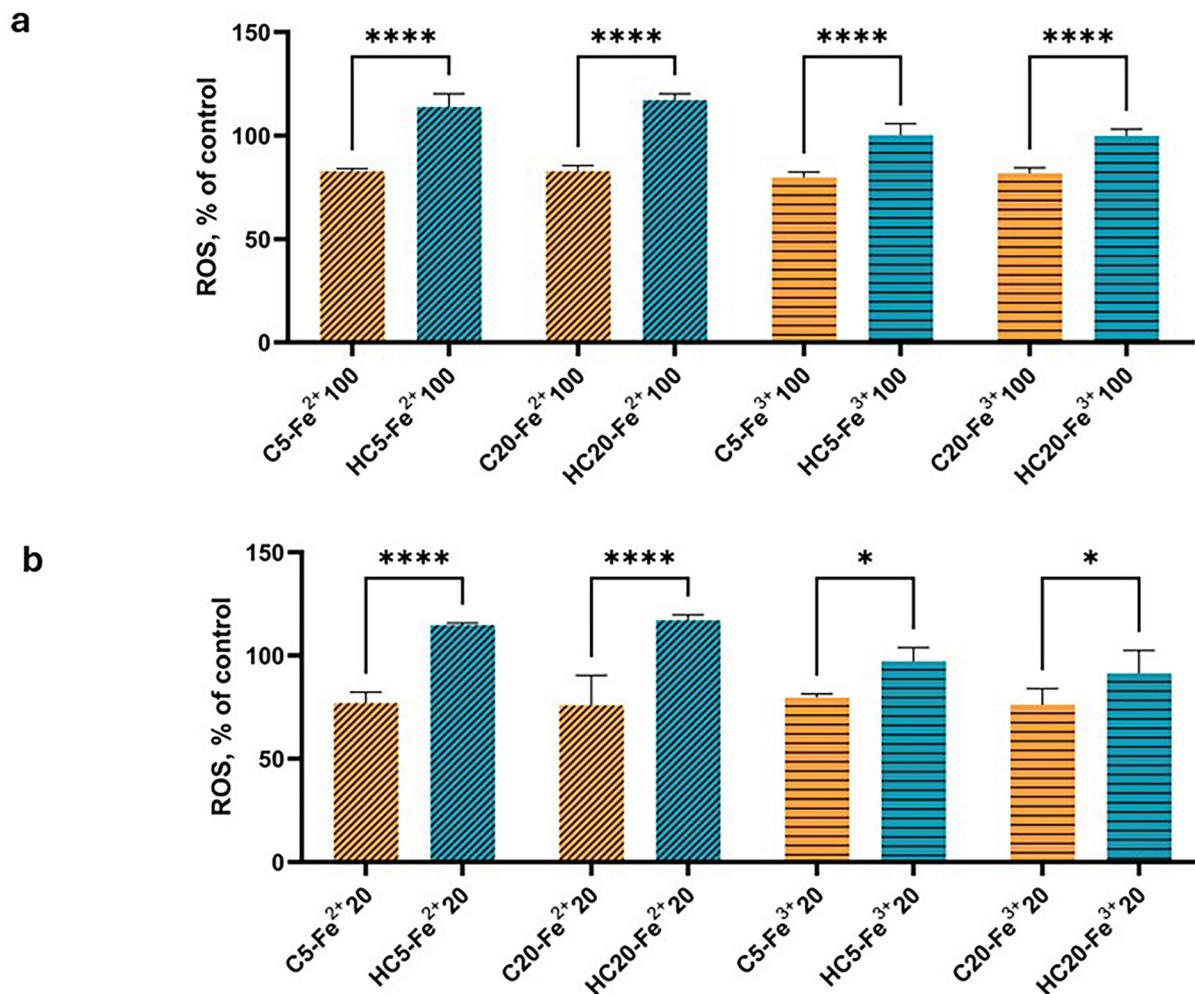


Fig. 5 ROS production in Caco-2 cells treated with 5 and 20 μM native curcumin (C) or formulated curcumin (HC) in combination with ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron at 20 μM (a) and 100 μM (b) concentrations. Values are expressed as mean \pm standard deviation of 8 replicates for control (MEM) and 4 replicates for test conditions. Data were analysed by one-way ANOVA and *post-hoc* Šidák's multiple comparisons test. * $P \leq 0.05$, *** $P \leq 0.001$, and **** $P < 0.0001$. Formulated curcumin significantly increased ROS production in Caco-2 co-treated with ferrous and ferric iron.



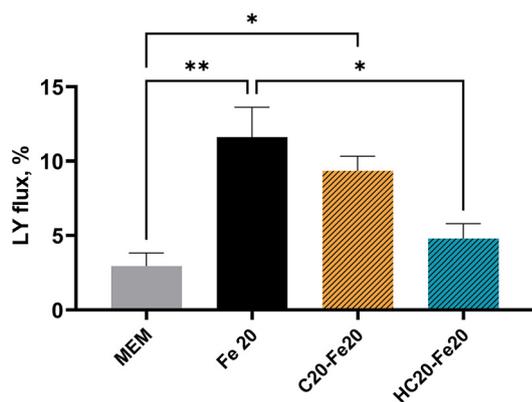


Fig. 6 Permeability of tight junctions by analysis of the flux of lucifer yellow (LY) probe across the Caco-2 monolayers following 24h treatment with 20 μ M ferrous iron (Fe 20) alone and combined with either 20 μ M native curcumin (C20) or 20 μ M formulated curcumin (HC20). MEM was used as control. Data are expressed as mean \pm standard deviation of 3 replicates. Data was analysed by one-way ANOVA followed by *post-hoc* Tukey's test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Iron increased the flux of LY across the monolayers but in presence of the formulated curcumin permeability reduced.

changes in the permeability of the tight junctions ($F(3,4) = 19.00$, $p = 0.0079$, Fig. 6). 20 μ M iron augmented the flux of Lucifer Yellow (LY) compared to control (11.61 ± 2.01 vs. 2.96 ± 0.86 , $p = 0.0090$). Furthermore, 20 μ M formulated curcumin significantly reversed this effect to $4.79\% \pm 1$ ($p = 0.212$, Fig. 6). The flux of LY across the monolayers treated with 20 μ M native curcumin and 20 μ M iron was 9.3 ± 0.96 , similar to monolayers treated only with iron ($p = 0.41$, Fig. 6).

Discussion

This study aimed to provide for the first time mechanistic data about the *in vitro* interaction between native and formulated forms of curcumin and iron and their potential link to iron uptake and intestinal cellular health by using the Caco-2 cell line as it is a well-established and validated *in vitro* model for iron uptake and permeability studies.^{54,57,58,68} HydroCurc® enhanced the uptake and storage of iron and demonstrated protection of the intestinal mechanical barrier against iron-induced damage of the tight junctions. While these effects may be attributed to HydroCurc®'s greater dispersibility and improved bioavailability compared to native curcumin,⁴³ our data suggests that the higher ferric reducing power of HydroCurc® may also play a role with regards to iron internalisation by the duodenal cells. Aqueous solutions of ferrous iron tend to oxidise to ferric iron at $pH > 3.5$ in the presence of oxygen.^{70,71} In the slightly acidic ($pH 5.8-6$) environment of the duodenum,⁵⁵ the main site for iron absorption,⁷² oxidation to ferric iron may occur. However, cells predominantly uptake ferrous iron *via* the divalent metal transporter 1 (DMT1) and possess the membrane ferrioreductase (DcytB) located on the brush border to catalyse the reduction of ferric iron to ferrous

form for subsequent uptake.^{73,74} Based on our observations, HydroCurc® may reinforce this process, enabling greater amounts of bioavailable ferrous iron in the lumen to be transported into duodenal cells *via* DMT1. The FeROUTINE human clinical trial found that HydroCurc® did not impair supplemental oral iron absorption, nor did it lead to a change in serum ferritin levels *versus* placebo after a 6-week supplementation period in healthy volunteers.^{37,42} This is in contrast to data from previous *in vitro* and *in vivo* research that indicated that curcumin may impair iron status.^{45,46,75} This may suggest that the formulation of curcumin could account for these differences. In line with this, HydroCurc® was shown to have a greater ferric iron reducing power than native curcumin, which might have allowed more readily absorbable ferrous iron, explaining the higher ferritin concentration in cells treated with HydroCurc®. Notably, the present data indicated that both native and formulated curcumin chelated substantially more ferric iron than ferrous iron. In this regard, it is worth noting that the investigations concluding curcumin iron-depleting properties were carried out with ferric forms of iron.^{45,46,75} Thus, the form of curcumin (native or formulated) as well as the type of iron salt (ferrous or ferric) may influence the net effect on body iron status. Finally, iron depletion was reported in studies where rodents ingested high levels of curcumin relative to iron (calculated molar ratio curcumin:iron ranging from 11:1 to 600:1) for 6 months.^{45,46} Importantly, it was shown that the influence of curcumin (12.5, 25 and 50 μ M) on ferritin in epithelial liver cells was reversed upon co-administration with iron (200 μ M), suggesting that co-administration with oral iron at a concentration higher than curcumin could attenuate the influence of curcumin on iron status.⁴⁶ In the FeROUTINE trial, the ratio of curcumin to iron was only 4:1 and 1:1.^{37,42} Therefore, the proportion in which curcumin and iron are co-administered could also impact iron absorption and status. It is worth mentioning that iron homeostasis is governed by hepcidin, a hormone that prevents dietary iron absorption in response to increased iron levels and inflammation.^{73,76} Hence, the effect of curcumin forms, native or formulated, on iron status will ultimately depend on this phenomenon. Indeed, a Caco-2/HepG2 cell model has been proposed for *in vitro* iron absorption studies.⁷⁷

Ferrous iron is linked to the oxidative stress that supplemental iron generates. This form of iron initiates the Fenton and Haber-Weiss reactions, leading to the production of hydroxyl radical, the most reactive ROS.^{14-16,78,79} Since HydroCurc® is effective in maintaining iron in ferrous form, a greater cellular ROS production upon co-treatment with HydroCurc® and ferrous iron was expected. The fact that ROS levels in cells treated with native curcumin were similar regardless of the form of iron co-administered further supports the idea that ferrous iron solutions oxidise to ferric upon preparation in cellular media used in our experiments ($pH 5.8-7.4$) and, more importantly, that HydroCurc®'s ferric iron reducing activity prevented to some extent this phenomenon. Notably, despite these observations, HydroCurc®-iron combinations' net effect on intestinal cellular viability did not seem to be



compromised, as indicated by our 24h cell viability data. Therefore, the initial burst of ROS production following 1 h treatment may be a transient effect that dissipates over a period of 24 hours partly due to the iron being gradually stored as ferritin within the intestinal cells. In addition, the temporary increase in ROS may also be overridden by curcumin's antioxidant activity *via* activation of Nrf2 (nuclear factor erythroid-derived 2-like 2), which upregulates the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and heme oxygenase (HO)-1.^{80–83} Indeed, following a 6-week supplementation period, HydroCurc® was shown to improve markers of oxidative stress in participants undertaking oral iron therapy,³⁸ suggesting that longer exposure to HydroCurc® may evoke positive effects on net redox balance.

Our data indicated that although native curcumin improved the permeability of the monolayers to some extent, it was insufficient to fully restore the integrity of the tight junctions. By contrast, HydroCurc® significantly reversed the iron-induced permeability, suggesting a potential to mitigate oral iron therapy-induced GI damage. The detrimental effect of iron on the permeability of Caco-2 monolayers has been associated with the activation of NF-κB, a pivotal mediator of inflammation.⁵³ Interestingly, curcumin can improve the integrity of Caco-2 monolayers *via* modulation of NF-κB, among other pathways.⁸⁴ As HydroCurc® has demonstrated superior bioavailability *versus* native curcumin,⁴³ greater amounts of curcumin could be internalised by the cells potentially leading to a greater inhibition of NF-κB and better protection of the tight junctions against iron damage. Assessment of the changes in the paracellular flux of Lucifer yellow probe through Caco-2 monolayers represents a sensitive technique to detect disruption of the tight junctions to help predict early damage to the intestinal membrane *in vivo*.²⁰ However, co-culture of Caco-2 with cells that produce mucins such as HT29-MTX has also been used to simulate the intestinal mucosal lining.

In sum, our data indicates that HydroCurc® may improve iron uptake by intestinal cells which could be partly due to its greater capacity to prevent the oxidation of the readily absorbable ferrous iron *versus* standard curcumin. Furthermore, HydroCurc® was observed to protect against iron-induced damage to the intestinal barrier. This suggests that this form of curcumin could potentially enhance iron absorption and reduce GI adverse events associated with supplemental ferrous iron. While the current data provides unique insights into the effect of native and formulated curcumin forms on oral iron uptake and prevention of associated GI events, there are limitations to this work. Our experiments focused primarily on gaining novel preliminary insights into the effects of the combinations of iron and curcumin forms on iron uptake and intestinal cellular health. While cell viability assays were conducted to assess the suitability of the treatments under experimental conditions more comprehensive examinations of their impact on cellular metabolism are warranted and can be the focus of further work.

Further research could broaden our current understanding of the role of curcumin forms in the protection against not only iron-induced oxidative stress but also dysbiosis, as well as

iron homeostasis. In this regard, analysing the impact of curcumin forms on ROS production at longer timepoints and exploring the effects on cellular antioxidant enzymes should be investigated in the future. Additionally, further investigations on the impact of co-administered iron and curcumin on intestinal permeability and iron uptake utilising Caco-2 co-culture models with mucin producing cells as well as cells secreting hepcidin would shed more lights on the cellular and extracellular dynamics.

Author contributions

Marta M. Gamez-Fernandez: conceptualization, formal analysis, investigation, methodology, project administration, resources, writing – original draft and editing. Helena Tiekou Lorinczova: investigation, resources, writing – review and editing. Stefanie Chan: investigation, resources. Satyanarayana Somavarapu: conceptualization, resources. Vinood B. Patel: conceptualization, methodology, supervision, writing – review and editing. Sanjoy K. Deb: supervision, writing – review and editing. Mohammed Gulrez Zariwala: conceptualization, funding acquisition, methodology, project administration, supervision, writing – review and editing.

Conflicts of interest

Marta M. Gamez-Fernandez received a scholarship from Pharmako Biotechnologies PTY Ltd to undertake a PhD from which this work arises. The rest of the authors have no conflicts of interest to declare.

Data availability

Data for this article are available at Harvard Dataverse data repository at <https://dataverse.harvard.edu/previewurl.xhtml?token=66c4f2f5-0254-4e39-ae6f-30f4ce7b7453>.

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