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Three Layers Formulation Improves Iron Gastrointestinal Bioavailability

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Abstract

The most common form of anemia is determined by iron deficiency in which the main problem is the low bioavailability of diet-derived iron. In this study, a new iron formulation (FerPlus Tre-Tard®, abbreviated as FerPlus), composed of three layers, and its ability to improve the bioavailability of iron during time was investigated in gastrointestinal cells *in vitro* model. GTL-16 and Caco-2 cell lines were used to evaluate the absorption of iron through stomach and intestinal epithelial barriers and in the first set of experiments, the effects of same iron concentration and the single agents for each layer were analyzed, In the second set of experiments, the effects of FerPlus were assessed in order to understand the absorption rate of iron during time. In addition, in Caco-2 model was also tested the FerPlus which crossed the GTL-16 barrier. Finally, in the third set of experiments, the effects of FerPlus were analyzed to explain the extrusion mechanism.

FerPlus showed a slow release in the stomach and it seemed to have an effect already in the stomach, which could be important to increase the amount of circulating iron. Moreover, the pre-digestion of FerPlus increased the intestinal absorption of iron. These data confirm the safety of the product, both in the pre-digested model and in the treatment with intact FerPlus.

Keywords: DMT1; Gastrointestinal Absorption; Iron Bioavailability; Iron Deficiency; Oral Intake

Introduction

Anemia is clinically characterized by blood hemoglobin or hematocrit values under the reference range for the patients [1]. The most common form of anemia is determined by iron deficiency derived from nutritional disorder [2]. This condition affects a large number of children and women in reproductive age, as well, both in developing and in industrialized countries; over 30% of the world's population, are anaemic, over 50% due to iron deficiency, and, in poorer areas, this is frequently exacerbated by infectious diseases [2]. Several factors can contribute to iron deficiency, for example the oxidant and anti-oxidant balance impairment, with elevation of levels of oxidative compounds [3]. Another important problem is the low bioavailability of iron present in the diet [4], which is composed of both non-heme and heme forms, absorbed in the duodenum [5]. Non-heme iron accounts for more than 85% of the total iron in the diet. Nevertheless, it shows low bioavailability (2-7%) as it is strongly affected by several dietary factors [6]. For this reason, food fortification is considered as a measure to increase the iron intake in general population [7]. Phytates, phenolic compounds, and calcium have been shown to inhibit non-heme iron transport, while ascorbic acid can enhance non-heme iron uptake.

However, heme iron is considered to be highly bioavailable (10-20%), and it is less affected by meal composition. Heme is released from hemoglobin during digestion so that it can be taken up by the duodenal enterocytes. The intact iron porphyrin is transported across the brush border membrane by the Heme Carrier Protein 1 (HCP1) [8]. Once inside the cell, the iron is released, and it is then likely to enter the low molecular weight pool of iron. Presently, iron supplements are the best options for maintaining iron level in the body. However, not only the iron content, but also the bioavailability of iron for absorption largely depends on the dietary components [9]. Current information of dietary Fe(III) absorption suggests synergistic association between an intestinal ferrireductase (DcytB) and the apical Fe(II) transporter Divalent

Metal Transporter 1 (DMT-1) such that the enzyme reduces Fe(III) allowing its transport and absorption [10]. Indeed, the absorption of iron differs from heme and non-heme form in the initial uptake step; heme enters mucosal cells through the brush border membrane by endocytosis whereas non-heme is transported into the cell in the ferrous form, mainly by the carrier DMT1 [11]. Since inorganic iron absorption requires multiple mechanisms to cross the intestinal barrier, this iron form in the supplements need to be less the organic form to maintain the optimally absorption from intestine [12]. The part of iron trapped in the intestinal epithelial cells is then eliminated through the stools at the end of the life cycle of the enterocyte [13]. In this context most iron supplements are made up of ferrous salts and are glycinate, sulfate, gluconate, and fumarate. Unfortunately, iron dietary supplementation is associated with potentially dangerous side effects and overload risk, if it is incorrectly absorbed. In addition, at gastric level some additional side effects are also present, such as hemorrhagic lesion [12]. In this context, new iron formulation composed of three layers improve the bioavailability of iron during time preventing the negative effects using a slowdown release of iron, was investigated on gastrointestinal *in vitro* model

Materials and Methods

Cell Culture

GTL-16 cell line donated by the Laboratory of Histology of the University of Eastern Piedmont, was cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich, Milan, Italy) supplemented with 10% Foetal Bovine Serum (FBS, Sigma Aldrich, Milan, Italy), 1% penicillin-streptomycin in incubator at 37 °C, 5% CO_2 [14]. The human intestinal Caco-2 cell line, purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), was grown in Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM-F12, Sigma-Aldrich, Milan, Italy) containing 10% Foetal) Bovine Serum (FBS, Sigma-Aldrich, Milan, Italy), 2 mM L-glutamine (Sigma-Aldrich, Milan, Italy), and 1% penicillin-streptomycin (Sigma-Aldrich, Milan, Italy) at 37 °C in incubator at 5% CO_2 [15]. Both cell types were plated for different protocols: 1 x 10^4 cells in 96-well plates to study cell viability by MTT test (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); 2 x 10^4 cells on 6.5 mm transwell with 0.4 µm pore polycarbonate membrane insert (Sigma-Aldrich, Milan, Italy) in a 24-well plate to investigate the absorption; 1x10^6 cells in 6-well plates to study the iron mechanisms. Before stimulations, both cell types were synchronized by incubation overnight in DMEM without red phenol (Sigma-Aldrich, Milan, Italy) and FBS and supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate in an incubator at 37 °C, 5% CO_2, and 95% humidity. GTL-16 cells plated on transwell insert were maintained in complete medium changed every other day, first basolaterally and then apically for 7 days before the stimulations [16,17]. Caco-2 Cells plated on transwell insert were maintained in complete medium changed every other day, first basolaterally and then apically for 21 days before stimulations [18,19].

Agent Preparations

FerPlus Tre-Tard® (FerPlus, Biosline, Padova, Italy) is a dietary supplement which is composed of three organic iron forms stratified in three level to improve the iron bioavailability with slow absorption. Each capsule is composed of 10mg iron Bysglicinate (BIS), 10mg iron Fumarate (FUM) and 10mg iron Pidolate (PID) supplemented with 180mg vitamin C (vitC), 18µg vitamin B12 (B12), 400µg Folic Acid (FA) and 75mg Beta-Carotene (BC). FerPlus was grinded and dissolved directly in the DMEM without red phenol and FBS but supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate to obtain a final concentration of 50 µM iron, as reported in literature [20]. In addition, all single agents were also tested to verify the mechanisms involved dissolving each of them directly in the medium to obtain a final concentration of: 200µM vitamin C [21]; 1.12µM vitamin B12 [22]; 1µM folic acid [23]; 20nM beta-carotene [24].

Experimental Protocol

Both cell lines were used to evaluate the absorption of iron through stomach and intestinal epithelial barriers, in order to clarify the mechanisms after oral intake. Finally, since the effectiveness of iron after oral intake depends on its composition, all single agents of the formulation were analyzed. The study was composed of three parts: in the first part, the effects of same iron concentration (50 µM) [20] and the single agents for each layer for different times (ranging from 1 h to 4 h in GTL-16 and from 1h to 6h in Caco-2 cells) were analyzed, in order to clarify the kinetics of absorption. In the second part, the effects of FerPlus were assessed, in order to understand the absorption rate of iron during time (ranging from 1 h to 4 h in GTL-16 and from 1h to 6h in Caco-2 cells). In addition on Caco-2 model was also tested the FerPlus which was cross to GTL-16 barrier. Finally, in the third part, the effects of FerPlus were analyzed at the end of stimulation period in order to explain the extrusion mechanism.

MTT Test

At the end of treatments, Caco-2 and GTL-16 cells were incubated with 1% MTT dye (MTT-based *In vitro* Toxicology Assay Kit, Sigma-Aldrich, Milan, Italy) in DMEM without red phenol 0% FBS for 2 h at 37 ˚C in incubator to analyze cell viability which was determined measuring absorbance at 570 nm with correction at 690nm through a spectrometer (VICTORX4 multilabel plate reader, PerkinElmer, Waltham, MA, USA) [14]. The results were obtained comparing the data to control cells (baseline 0) [25].

GTL-16 Permeability Assay

GTL-16 cells plated on 24-well transwell inserts at 7 days were fully confluent, so experiments were initiated at that time. The effects of FerPlus absorption on apical-to-basolateral (Ap-Bl) was investigated at pH gradients. For this reason, the medium was changed on apical (donor compartment) side to obtain pH 3 adding HCl to the medium for 60 min, following as previous described [16,17]. After this time, the stimulation was performed ranging from 1h to 4h, to mimic the digestion time, and then iron quantity was measured by kit.

Caco-2 Permeability Assay

Caco-2 cells plated on 24-well transwell inserts after 21 days were cultured under different pH conditions, as reported in literature [18,19], with acidic pH (pH 6.5) in the apical side and the neutral pH (pH 7.4) in the basolateral side. In this condition, FerPlus was added to the apical environment and during treatments the cells were maintained in incubator at 5% CO_2. At each time point the iron quantity was measured by iron assay kit.

Iron Quantification Assay

Total iron was measured in the samples using Iron Assay Kit (Sigma-Aldrich, Milan, Italy) following the manufacturer's instructions. At the apical side of transwell, Caco-2 and GTL-16 cells were lysed in 4 volumes of cold Iron Assay Buffer and at the basolateral medium was directly measured [12]. 5 µL of Iron Reducer were added at all samples to reduce Fe^{3+} to measure total iron. All reactions were incubated for 30 min at Room Temperature (RT), protected from light and then 100 µL of Iron Probe were added to each well and incubated for 60 min at RT and protected from light. The absorbance was measured by spectrometer (VICTORX4 multilabel plate reader, PerkinElmer, Waltham, MA, USA) at 593 nm. Total iron (Fe^{2+} + Fe^{3+}) concentrations can be determined from the standard curve and the iron concentration was expressed as ng/µL.

Western Blot of Cell Lysates

Caco-2 and GTL-16 cells were lysed in ice Complete Tablet Buffer (Roche, Milan, Italy) supplemented with 2 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich, Milan, Italy), 1:50 mix Phosphatase Inhibitor Cocktail (Sigma-Aldrich, Milan, Italy) and 1:200 mix Protease Inhibitor Cocktail (Calbiochem, San Diego, USA). 35 µg of proteins of each sample were resolved on 10% SDS-PAGE gels and polyvinylidene difluoride membranes (PVDF, GE, Healthcare Europe GmbH, Milan, Italy) were incubated overnight at 4 °C with specific primary antibody: anti-ferroportin (1:250, Santa Cruz Biotechnology, Dallas, USA), anti-ferritin (1:250, Santa Cruz Biotechnology, Dallas, USA) and anti-DMT1 (1:250, Santa Cruz Biotechnology, Dallas, USA). Protein expression was normalized and verified through β-actin detection (1:5000; Sigma-Aldrich, Milan, Italy) and expressed as mean ± SD (% vs. control).

Statistical Analysis

The results are expressed as means ± SD of four independent experiments performed in four technical replicates. One-way ANOVA followed by Bonferroni post hoc test were used for statistical analysis, and pairwise differences compared by Mann-Whitney U tests. p-values < 0.05 were considered statistically significant.

Results

Cell Viability in Time-Course Study Under Treatments with FerPlus and its components on GTL-16 cells

In order to exclude any harmful event or damage to the epithelial gastric cells during treatments with FerPlus and its components, in the first set of experiments cell viability by MTT was investigated. The emptying time of stomach is usually around 4 hours in normal conditions of full stomach and around 2-3 hours in empty stomach condition. Therefore, cell viability was tested on GTL-16 cell during 4 hours (starting from 1 hours to 4 hours) under treatments with FerPlus and its components, to mimic the emptying time of the stomach. As shown in Figure 1, the single ferric components exerted different effects on cell viability during time; BIS showed a greater effect starting from 1 hour to 2 hours (Figure 1A) compared to control ($p<0.05$) and to other time ($p<0.05$). Conversely, FUM showed the maximum effect on cell viability after 3 hours (Figure 1B) compared to control (about 9%) and to all other times ($p<0.05$); finally, PID exerted the maximal effect on cell viability after 4 hours (Figure 1C) of treatment compared to control (about 18%) and to all other time ($p<0.05$). These times of effects reflected the construction of FerPus, in which the iron layer may be dissolved and acted in different times to obtain a slow iron absorption. Indeed, FerPlus (Figure 1D) caused a significant increase in cell viability compared to control starting from 2h ($p <0.05$) and this effect remains significant over time (even at 4h) with a greater effect at 4 hours compared to control ($p<0.05$) and to all other time ($p<0.05$) and to the single ferric components taken at the same time (5, 11 and 1 folder to BIS, FUM and PID respectively).

Figure 1

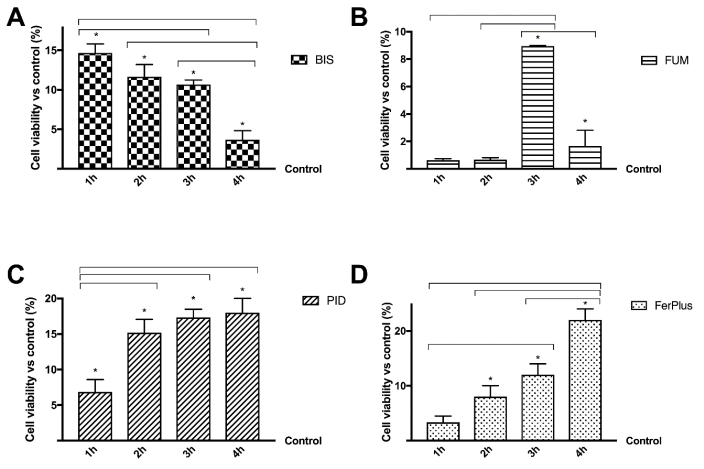

Figure 1: Cell viability on GTL-16 cells under treatments with FerPlus and its iron form. In A BIS, in B FUM and in C PID effects measured by MTT test during time ranging from 1-4h. In D FerPlus effect measured by MTT test in a time course study. BIS= iron bysglicinate, FUM= iron fumarate, PID= iron pidolate. Data are expressed as means ±SD (%) of four independent experiments performed in triplicates normalized to control (0 line as control). * p<0.05 vs control; bars p<0.05 between time of stimulations.

These findings allow to hypothesize that the slow release of FerPlus in the stomach permits its use without significant negative effects. In addition, FerPlus seems to have an effect already at the level of the stomach, which could be important to increase the amount of circulating iron. Since to improve the release of iron from FerPlus, some additional components were used to verify that each agent were also tested. As shown in (Figure 2), the different agents of FerPlus show a positive effect on cell viability during time. VitC showed a greater effect after 3 hours (Figure 2A) compared to control (p<0.05) and to other time of stimulation; B12 exerted a main effect after 2 hours (Figure 2B) compared to control (p<0.05); FA acted on cell viability in a time-dependent manner with the main effect starting from 3 hours to 4 hours (Figure 2C) compared to control (p<0.05) and to other time (p<0.05) of stimulation; BC showed the maximal effect within 1 hour (Figure 2D) compared to control (p<0.05) and to other time (p<0.05) and remain significant till to 3h compared to control (p<0.05). Every agent seems to support in the different moments the release of iron from FerPlus.

Figure 2

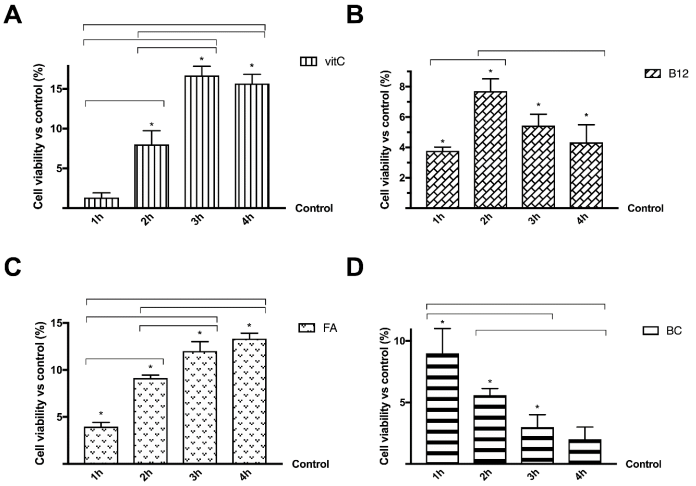

Figure 2: Cell viability on GTL-16 cells under treatments with the additional components. In A vitC, in B B12, in C FA and in D BC effects measured by MTT test during time ranging from 1-4h. vitC= vitamin C, B12= vitamin B12, FA= folic acid, BC= beta-carotene. Data are expressed as means ±SD (%) of four independent experiments performed in triplicates normalized to control (0 line as control). * p<0.05 vs control; bars p<0.05 between time of stimulations.

Cell Viability Under Treatments with FerPlus and Its Components On Caco-2 Cells

Since iron crosses gastric barrier to reach intestinal barrier, also in this district may be have an irritative action, the effects of FerPlus and its components were evaluated on human intestinal epithelial cells. The emptying time of intestine ends within 6 hours, therefore, the analysis of viability was carried out at 1h, 2h, 3h, 4h, 5h, 6h to mimic the duration of emptying of the intestine and the maximum absorption time of molecules. As shown in (Figure 3), the influence on cell viability exerted by the single ferric components was different during time; BIS showed the maximal effect at 2 hours (Figure 3A) compared to control (p<0.05, about 48%) and to other times of stimulation (p<0.05); FUM exhibited a greater effect at 3 hours (Figure 3B) compared to control (p<0.05, about 56%) and to other times of treatments (p<0.05); PID had a time-dependent effect starting from 1h to 4h, in which was showed the main effect (Figure 3C) compared to control (p<0.05, about 44%) and to other times (p<0.05) of administration. These findings support the slow release of FerPlus composition. Indeed, FerPlus

(Figure 3D) appeared to have higher effects than the single ferric components, with a greater effect at 4h of stimulation compared to control ($p<0.05$, about 66%) and to other times ($p<0.05$). Since FerPlus is made up of other active agents, in order to exclude any inside effect, all of these agents were also investigated in the same manner. As shown in (Figure 4), there are no contraindications in the use of the other agents present in FerPlus; indeed, each component seems to support the release of iron from FerPlus. VitC showed a time dependent increase in cell viability starting from 1h to 4 hours, which correspond to the main effect (Figure 4A) compared to control ($p<0.05$) and to other times ($p<0.05$); B12 exerted a greater effect at 3 hours (Figure 4B) compared to control ($p<0.05$, about 61%); FA showed a significant increase in cell viability at 4 hours (Figure 4C) compared to control ($p<0.05$, about 8%); BC was able to induce a greater effect after 3 hours of stimulation (Figure 4D) compared to control ($p<0.05$, about 35%) and to other times ($p<0.05$). These data allow to exclude any negative responses from intestinal epithelium with FerPlus stimulation.

Figure 3

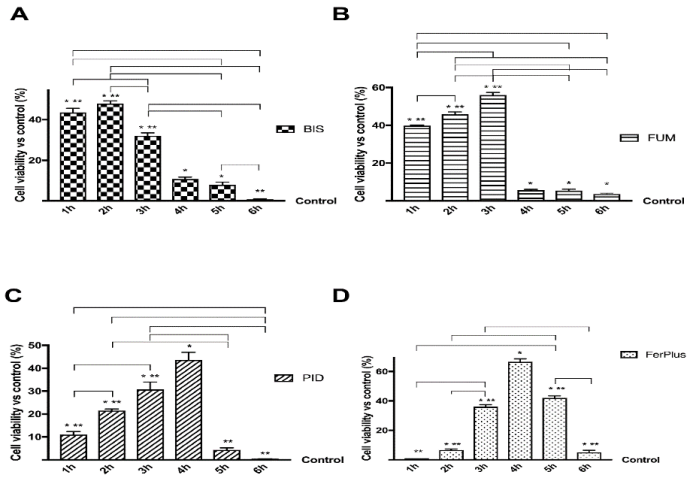

Figure 3: Cell viability on Caco-2 cells under treatments with FerPlus and its iron form. In A BIS, in B FUM and in C PID effects measured by MTT test during time ranging from 1-6h. In D FerPlus effect measured by MTT test in a time course study. The abbreviations are the same reported in (Figure 1). Data are expressed as means ±SD (%) of four independent experiments performed in triplicates normalized to control (0 line as control). * $p<0.05$ vs control; bars $p<0.05$ between time of stimulations.

Figure 4

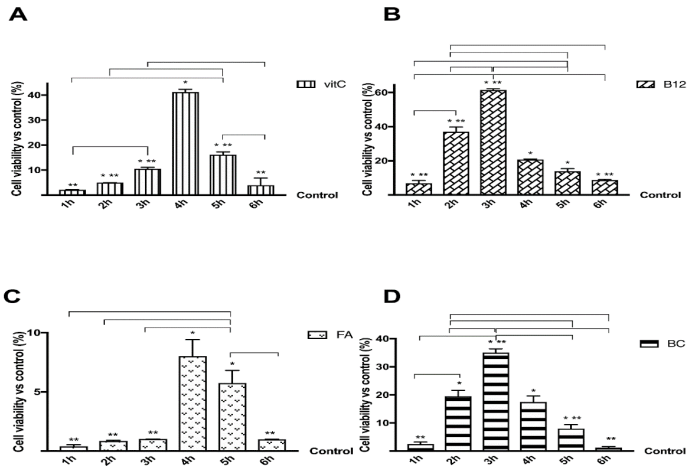

Figure 4: Cell viability on Caco-2 cells under treatments with the additional components. In A vitC, in B B12, in C FA and in D BC effects measured by MTT test during time ranging from 1-6h. The abbreviations are the same reported in (Figure 2). Data are expressed as means ±SD (%) of four independent experiments performed in triplicates normalized to control (0 line as control). * $p<0.05$ vs control; bars $p<0.05$ between time of stimulations.

Gastrointestinal Absorption of FerPlus

Since in gastric and intestinal epithelial cells were observed a significant increase of cell viability after stimulation with FerPlus, the absorption of iron may be hypothesized already at stomach level. This is important because it can be hypothesized a faster passage to the blood, before the intestinal barrier. An *in vitro* gastric barrier model has been performed to measure the permeability of the gastric barrier after FerPlus treatment. As shown in (Figure 5A), a time dependent absorption measurement was carried out in a range from 1h to 4h (1, 2, 3, 4 hours), and at basolateral level (that simulates blood) the iron concentration was analyzed. A part of product released already in the stomach comes absorbed starting from 2h compared to control ($p<0.05$) and reaches a plateau phase between 3h and 4h (about 15% compared to the control). This data suggests that the absorbed portion is not retained in the cytoplasm of the cells but is directly put into circulation. In addition, to understand the mechanism of FerPlus, an *in vitro* intestinal barrier model has been created using Caco-2 cells to measure the permeability of the intestinal barrier after treatment with

FerPlus pre-digested or not by GTL-16 cell. This data is important for determining the amount of iron that reaches the blood to be bioavailable for the body. The iron concentrations after stimulation with intact FerPlus and pre-digested FerPlus (obtained from apical portion of stomach barrier) taken at different times (2h, 3h and 4h) were evaluated. As shown in (Figure 5B), FerPlus was able to cross the intestinal barrier starting from 1h and it is maintained over time with maximum absorption time in 4-5h ($p<0.05$). Instead, the pre-digested products were more absorbed than FerPlus not pre-digested supporting the active and physiological role of absorption following gastric level. A pre-digestion of FerPlus for 3h increases the absorption of iron, indicating that it is not absorbed in the stomach but it is absorbed by the enterocyte. In terms of bioavailability FerPlus has two equivalent moments of absorption that are at the gastric level and then at the intestinal level which cover a long time frame, thus offering better coverage over time and greater bioavailability. These data confirm the safety of the product, both in the pre-digested model and in the treatment with FerPlus not pre-digested.

Figure 5

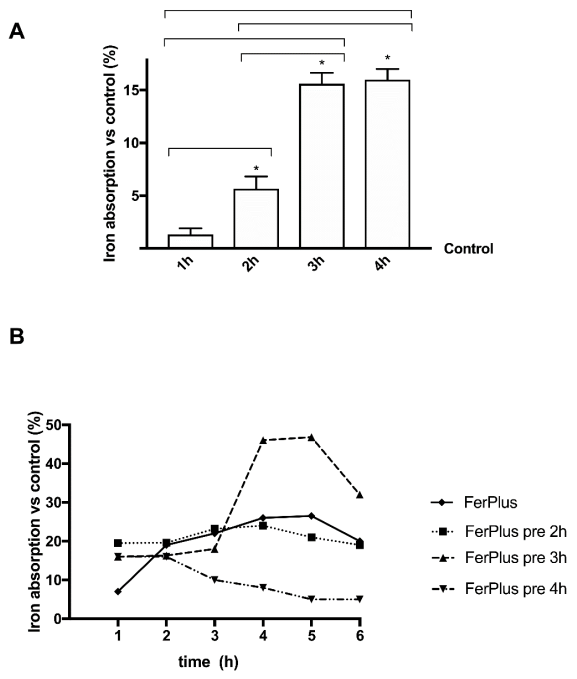

Figure 5: Iron total quantification on gastric and intestinal barrier in *in vitro* model. In A iron presents at basolateral level during time in gastric model; in B the same measurement obtained on intestinal barrier during time. In intestinal model the effects of FerPlus digested were also reported. FerPlus pre= FerPlus digested by GTL-16 cells. Data are expressed as means ±SD (%) of four independent experiments performed in triplicates normalized to control (0 line as control). In A * $p<0.05$ vs control; bars $p<0.05$ between time of stimulations. In B all data $p<0.05$ vs control.

Study of Intracellular Pathways Activated by FerPlus On Gastrointestinal System

As previously described the main absorption time of FerPlus at gastrointestinal level is at 4 hours of stimulation, the role of DMT1 (the iron transporter), Ferritin (the cytoplasmic store of iron) and Ferroportin (the transmembrane transporter) mediators were also investigated on both cell types. These mediators are important to analyze the mechanism of iron once it has entered the cell to exclude that in part it remains trapped in the cytoplasm. Iron trapped can create oxidative conditions and in chronic use leads to cell damage. As shown in (Figure 6A) at gastric level, DMT1 is significantly activated at 4h ($p<0.05$) compared to control and compared to 2h of treatment with FerPlus; Ferritin (Figure 6B) is not activated at 2h and at 4h confirming the ability of FerPlus to cross the barrier; finally, the activation of Ferroportin (Figure 6C), which is significantly increased at 4h ($p<0.05$) compared to 2h and to control, indicated that iron is not retained inside the cell.

Figure 6

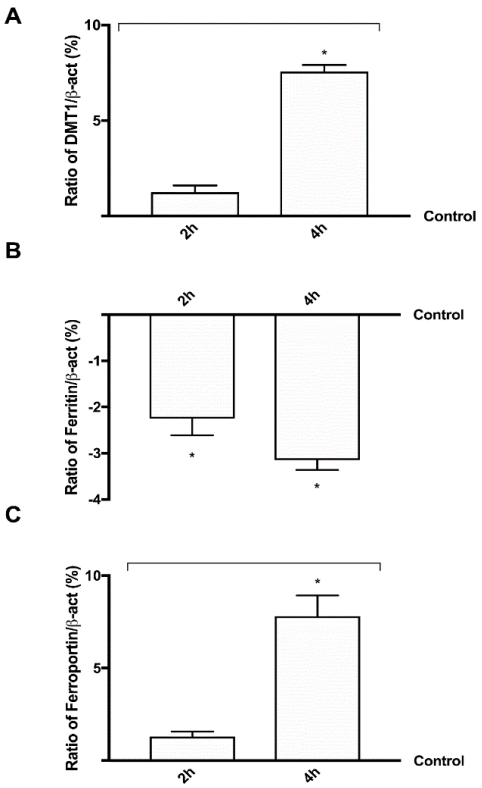

Figure 6: Densitometric analysis of FerPlus on GTL-16 cells of the iron metabolism markers. In A DMT1, in B Ferritin, in C Ferroportin expressions measured by Western blot at 2h and 4h. Data are expressed as means ±SD (%) of four independent experiments performed in triplicates normalized and verify on beta-actin detection.* $p<0.05$ vs control; bars $p<0.05$ between 2h and 4h of stimulation.

These data confirm that the treatment with FerPlus allows to have a blood bioavailability in the first hours after oral administration at the gastric level. Since the main absorption rate was observed at 4h also in intestinal cells, the role of DMT1 (the iron transporter), Ferritin (the cytoplasmic store of iron) and Ferroportin (the transmembrane transporter) were also analyzed in presence of intact FerPlus and pre-digested FerPlus for 3h, on Caco-2 cells. As shown in (Figure 7A), DMT1 is significantly expressed at 4h ($p < 0.05$) both in pre-digested and not predigested samples, compared to control ($p<0.05$), confirming the ability of FerPlus to cross inside the cells. Moreover, this amount of iron is immediately excreted, as well confirmed by Ferritin expression, which is maintained at basal level (Figure 7B) on both pre-digested and not pre-digested samples, compared to control ($p<0.05$). This secreting mechanism of iron was confirmed by the analysis of Ferroportin (Figure 7C), which resulted significantly increased ($p < 0.05$) compared to control in both samples and the FerPlus pre-digested seem to be a greater effect compared to intact FerPlus ($p<0.05$). All these data confirm that the treatment with FerPlus allows a better bioavailability during time.

Figure 7

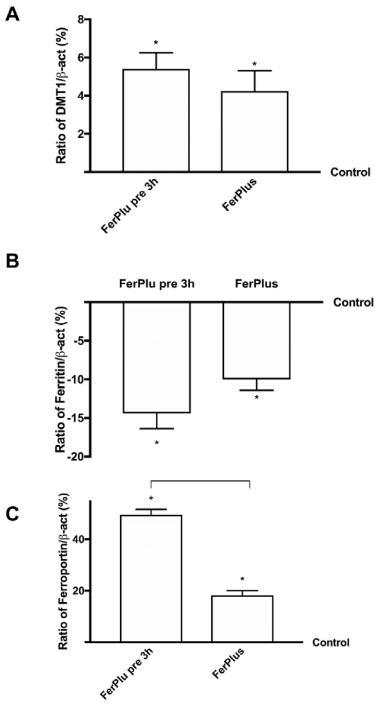

Figure 7: Densitometric analysis of FerPlus on Caco-2 cells of the iron metabolism markers. In A DMT1, in B Ferritin, in C Ferroportin expressions measured by Western blot at 4h. Data are expressed as means ±SD (%) of four independent experiments performed in triplicates normalized and verify on beta-actin detection.* $p<0.05$ vs control; bars $p<0.05$ between pre or not digested.

Discussion

Iron deficiency is the predominant cause of anemia that affects one-fourth of the world's population. Patients with iron deficiency with unknown etiology are frequently referred to a gastrointestinal disorder which is the major cause of iron deficiency. Current strategies to restore the level of iron in the body, includes treatments based on oral ferrous iron supplementation [20]: however, the efficacy of oral iron intake is limited in different gastrointestinal conditions, such as inflammatory bowel disease, celiac disease, and autoimmune gastritis [26]. Intestinal iron absorption is limited. The maximum rate of absorption of 100 mg after oral intake is about 20-25% and it is reached only in the late stage of iron deficiency. Latent iron deficiency and iron deficiency anemia correspond to mean absorption rates of 10% and 13%, respectively, whereas healthy males absorb 5% and healthy females 5.6% [27]. Iron that remains in the intestinal lumen or at gastric level may cause epithelial injury, [28,29] and studies in animal models suggest an exacerbation of disease activity and the induction of carcinogenesis in IBD (inflammatory bowel disease) [30]. Moreover, the iron formulation can be very important in determining such side effects as severe corrosive injury to the epithelial gastrointestinal cells, including mucosal necrosis, ulceration, and ischemia [12]. The biocompatibility of iron dietary supplements is a common issue in treatment of iron deficiency anaemia. On a chemical basis, dietary iron is classified as non-heme iron (inorganic ferric or ferrous iron) and heme iron. Ferritins are ubiquitous iron storing proteins present in animals and it is likely that ferritin-bound iron is less sensitive to chelators such as phytates present in the diet and, therefore, forms a bioavailable source of iron [7]. In this study, we found that bioavailability of iron was significantly enhanced by a particular formulation of a dietary iron supplement, named FerPlus, made of three layers able to induce a slowed release. For the first time the importance of the Fe physiology by FerPlus was demonstrated analyzing the effects at gastric and intestinal level. It is also noteworthy that the gastric digestion model and emptying *in vitro* and as well in humans is a dynamic process. Therefore, the released ferritin iron has equal chances of interaction with either enterocyte in the food, and thereby the observed high bioavailability of ferritin iron in humans. However, the stomach has never been considered a tissue involved in iron absorption and it has received less investigations when proteins involved in iron metabolism have been localized. However, the non-iron-responsive element isoform of the DMT1 gene is expressed in the antral portion of the rat stomach [31], and both the iron-responsive and non-iron-responsive genes are expressed in the mouse stomach [32]. The stomach, therefore, may be another site of iron absorption, or perhaps the epithelial cells of the antral mucosa participate in a bacteriostatic function by reducing the amount of iron in the stomach [33]. Our results support the finding that iron contained in FerPlus in the acid

environment of the stomach lumen could transport iron into the cell by DMT1 after the digestion time. Therefore, in order to obtain the most relevant results that could be translated for future *in vivo* studies, each physiologically relevant sample was added to the Caco-2 experiments.

Since the Caco-2 cell line exhibits a phenotype similar to human small intestinal enterocytes, they has been extensively used for iron uptake studies [10]. Our data suggest that an endocytic, apical uptake mechanism exists in these differentiated Caco-2 cells for uptake of dietary supplement FerPlus. Iron released from FerPlus after digestion at gastrointestinal level, is able to across the apical membrane of enterocytes intact via the DMT1 transporter. The absorption is determined by the release of iron from the digesta and ionization during the first hours of digestion. Generally, when measuring non-heme iron absorption, a 2h dialysis step is included to determine how much ionic iron is released from the food matrix, as only ionic iron is considered to be bioavailable [6]. Finally, in gastrointestinal system, iron contained in FerPlus exerts a physiological effect to maintain during time a release of iron and to support the physiological blood concentration by the regulation of ferritin-protein and ferroportin turnover. Free Fe is potentially toxic to cells at gastric and intestinal level. So, the purpose of using FerPlus is that the cells lining the gut and stomach are protected from redox damage by a slowed release/absorption of iron during time according to the additional agents present in each layer. These phenomena are consistent with other aspects of Fe metabolism. For example, beta-carotene which is well known to improved iron absorption *in vivo* and *in vitro* model [34]. Our results showed for the first time that FerPlus bioaccessibility has two distinct and related phase: one gastric and the other small intestinal and they are both important to maintain blood iron level.

Conclusion

In conclusion, the bioavailability of iron was significantly improved by the use of FerPlus, a particular formulation made of three layers. Experimental findings demonstrated a slow release of iron that permits its administration without significant negative effects. In addition, in gastrointestinal system, iron contained in FerPlus exerts a physiological effect that consists in maintaining iron release over time to support the physiological blood concentration. FerPlus has a slow release within the stomach and so, it seems to have an effect already at the level of the stomach, which could be important to increase the amount of circulating iron. This pre-digestion phase of FerPlus can ultimately increase the intestinal absorption of iron.

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