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Ethylenediaminetetraacetic acid (EDTA) does not increase iron uptake or ferritin synthesis by Caco-2 cells

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Abstract

Ethylenediaminetetraacetic acid (NaFe-EDTA) is a chelator capable of binding a wide variety of metals, with a high affinity constant for Fe^{3+}. NaFe-EDTA has been extensively studied and validated as an excellent choice for iron fortification programs and extensive research has demonstrated its high bioavailability specially for cereal based foods. To further evaluate the usefulness of this compound we performed iron uptake experiments with EDTA using the Caco-2 cell system. Cells were incubated in PBS at pH 5.5 or 7.0, containing or not ascorbic acid. Different sources of EDTA, different concentrations of NaFe-EDTA and the inclusion of another iron compound as electrolytic iron, were tested. Also, the ferritin content of Caco-2 cells 24h after 1h incubation with iron compounds was evaluated. Except for the addition of ascorbic acid, under the experimental conditions used, Caco-2 cells were not capable of obtaining iron from NaFe-EDTA. Furthermore, iron uptake from electrolytic iron was inhibited when Na_2 or K_2-EDTA were included. Ferritin determinations to Caco-2 cells evaluated 24h after 1h incubation periods, showed that NaFe-EDTA did not induce new ferritin synthesis, since iron did not enter the cells. Further studies are required to evaluate incorporation of iron from NaFe-EDTA to a common iron pool and the requirements for iron uptake by Caco-2 cells.

Keywords: Iron uptake; Caco-2 cells; EDTA; Ferritin; Non-heme iron pool

1. Introduction

Ethylenediaminetetraacetic acid (EDTA) is a chelator capable of binding a wide variety of metals with a high affinity constant for Fe^{3+}, depending on the iron:EDTA molar ratio, the presence of competing metal ions and the pH [1].

Sodium iron ethylenediaminetetraacetic acid (NaFe-EDTA) has been studied and validated as an excellent choice for iron fortification programs and extensive research has demonstrated its usefulness and high bioavailability, specially for cereal based foods. In fact, there are 3 clinical trials in Guatemala [2,3], Thailand [4] and South Africa [5,6] that have demonstrated that NaFe-EDTA successfully reduces iron deficiency anemia.

Some of the advantages of this compound are its solubility in aqueous solutions and low toxicity [7,8]. It has been reported that iron availability from NaFe-EDTA is high, its absorption is at least double than from other iron compounds such as ferrous sulfate [2,9,10], especially from diets high in phytates such as cereals [11,12]. At the same time it has been corroborated that increased iron absorption from EDTA, does not imply a significantly lower absorption of other elements such as zinc, calcium or copper [13–15].

Another interesting feature of NaFe-EDTA is its capability of increasing iron absorption from other iron sources present in the same food. This effect was also reported with other forms of EDTA, such as Na_2 or Ca_2-EDTA [9,10,16].

When NaFe-EDTA is ingested with food, iron remains bound to the molecule in the stomach and liberated, mostly by exchange with other metal ions, in the alkaline milieu of the small intestine. Only a small fraction of EDTA is absorbed and excreted in the urine, while iron enters the non-heme iron pool to be absorbed [10,17–19].

To further evaluate the usefulness of this compound, we performed iron uptake experiments with EDTA using the Caco-2 cells, a human adenocarcinoma cell line that has been successfully used to study iron metabolism, since they differentiate in culture into polarized monolayers presenting characteristics of normal absorptive epithelium, such as brush border membrane and enzymes associated to normal enterocytes [20,21].

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Iron uptake studies with Caco-2 cells have demonstrated that this model responds in a way similar to human epithelium to changes in pH, iron concentration and to the iron compound administered and its oxidation state [22–25]. Moreover, iron uptake by Caco-2 cells responds in a similar way to inhibitors (such as tannic acid and phytates) and enhancers (such as ascorbic acid, meat and cysteine) of iron absorption in humans [26–29]. Recently, a report by Au & Reddy [30] shows that there is a high correlation between published human absorption data and iron uptake by Caco-2 cells.

The objective of this study was to evaluate iron uptake by Caco-2 cells from NaFe-EDTA and other iron compounds under different experimental conditions, and to measure their impact on ferritin synthesis. Also, the effect of EDTA as NaFe-, Na_2- or K_2-EDTA on uptake from electrolytic iron was also assessed.

2. Methods and materials

2.1. Cell culture

Caco-2 cells, a human colon adenocarcinoma cell line (HTB 37), were donated by Dr. F. Liprandi (Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela). Cells were grown in Falcon T-75 tissue culture flasks (Beckton-Dickinson, Lincoln Park, NJ) in Dulbecco's modified Eagle's medium with high glucose (DMEM) [2] plus 1 mmol/L nonessential amino acids, antibiotics/antimycotics, 2 mmol/L glutamine, 5 mmol/L HEPES, 0.4 mmol/L pyruvate and 15% fetal bovine serum (Gibco BRL, Gaithersburg, MD).

After reaching 80 to 90% confluence, cells were trypsinized and seeded into T-25 flasks (Beckton-Dickinson, Lincoln Park, NJ) in supplemented DMEM at a density of $5x10^4$ cells/cm^2 and fed every 2 days with supplemented DMEM. Experiments were performed at 16 days in culture, 1 week after the cells reached confluence. The integrity of the monolayer was tested by optic microscopy. All experiments were performed on passages 19 to 35.

2.2. Uptake experiments

Cells were rinsed with PBS and incubated at 37°C for 1 h with 7.5 mL of an incubation mixture containing PBS adjusted to pH 5.5 (unless otherwise indicated), 37 kBq ^{59}Fe as $FeCl_3$ (NEN Life Sciences Products, Boston MA) and the compounds and conditions to be tested. After 60 min incubation, media was removed, cells rinsed once with PBS containing 1 mM Na_2-EDTA, detached from flasks with trypsin-EDTA (0.5% trypsin +5.3 mM EDTA, Gibco BRL, Gaithersburg, MD) and counted in a Compugamma LKB gamma counter to determine iron uptake. Results were calculated as pmol Fe/mg cell protein (3.55 mg protein/flask) and also as percentage of uptake based on iron initially added to the incubation media (37 kBq for flasks).

2.2.1. a) Iron uptake from different iron compounds. Effect of ascorbic acid.

Incubation mixtures were prepared containing PBS adjusted to pH 5.5, 37 kBq ^{59}Fe and 3 μmol/L of iron as ferrous fumarate, ethylenediaminetetraacetic acid ferric-sodium salt (NaFe-EDTA), iron-amino acid chelate (Ferrochel) or electrolytic iron. This iron concentration is equivalent to approximately 25X the iron content of the isotope, with slight variations depending on the specific activity of ^{59}Fe. Ascorbic acid (AA) was added to some incubation media at 6 μmol/L (Fe: AA molar ratio 1:2).

2.2.2. b) Effect of pH.

Due to the low uptake values from NaFe-EDTA, some experiments were performed preparing incubation media at pH 5.5 and 7.5, adjusting the pH of PBS to those values. The iron compounds tested were ferrous fumarate, reduced iron, electrolytic iron and NaFe-EDTA. The pH of the incubation media was adjusted to the required value before addition of ^{59}Fe and iron compounds.

2.2.3. c) Iron uptake from increasing doses of NaFe-EDTA. Effect of this compound on uptake from electrolytic iron.

Increasing concentrations of NaFe-EDTA at 1.5, 3.0, 6.0 and 9.0 μmol/L, (corresponding from 0.5 to 3X the cold iron concentration used to perform uptake experiments) were tested. Other iron source, electrolytic iron at 3 μmol/L, was added to the incubation media containing NaFe-EDTA, to evaluate changes in uptake.

2.2.4. d) EDTA.

To evaluate iron uptake from NaFe-EDTA and the effect of other EDTA salts (K_2-EDTA and Na_2-EDTA) on uptake from electrolytic iron, incubation media was prepared adding the EDTA source at 14 μmol/L. For NaFe-EDTA, this concentration provided 3 μmol Fe/L. Addition of electrolytic iron, also at 3 μmol/L, was evaluated for each EDTA source. Additionally, iron uptake from ^{59}Fe alone was evaluated in each experiment.

2.3. Effect of different iron compounds on uptake and cellular ferritin concentration 24 h post-incubation.

For these experiments 2 sets of flasks were prepared, containing incubation media adjusted to pH 5.5, ^{59}Fe and the iron compound to be tested (ferrous fumarate, electrolytic iron, reduced iron or NaFe-EDTA at 3 μmol/L). Both sets of flasks were incubated for 1 h and rinsed with PBS. One set of flasks was treated as described before, that is, trypsinized and counted in a gamma counter. To the other group of flasks 2.5 mL of PBS pH 7.5 were added and cells were incubated at 37°C for 24 h. Cells were then detached

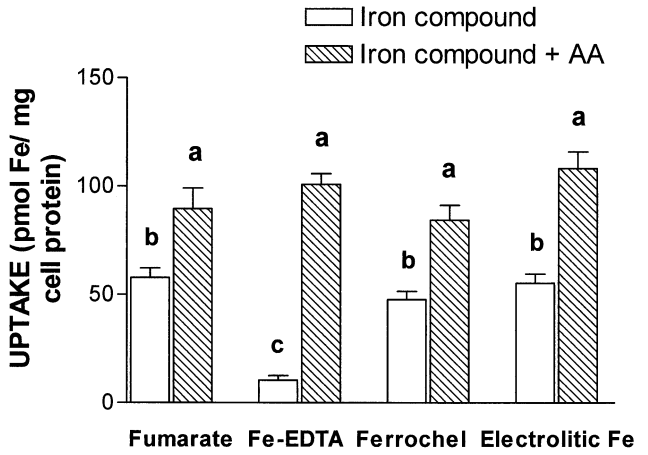

Fig. 1. Iron uptake from ferrous fumarate, NaFe-EDTA, Ferrochel and electrolytic iron by Caco-2 cells. Effect of ascorbic acid. Caco-2 cells were incubated with 7.5 mL PBS pH 5.5 containing 37 KBq ^{59}Fe, 3 μmol/L of iron as ferrous fumarate, NaFe-EDTA, ferrochel or electrolytic iron. Ascorbic acid was added at 6 μmol/L. After 1h incubation at 37°C, cells were rinsed with PBS-EDTA, trypsinized and counted to measure uptake. Values are means ± SD, n = 5. Means with no common letters differ $p < 0.05$.

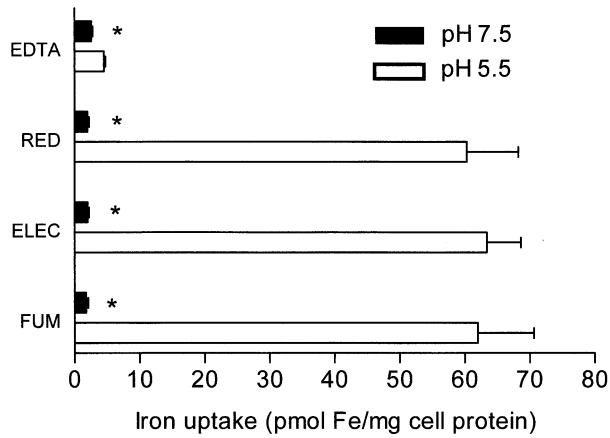

Fig. 2. Iron uptake from ferrous fumarate, electrolytic iron, reduced iron and NaFe-EDTA at pH 5.5 and 7.5. Caco-2 cells were incubated with 7.5 mL PBS adjusted to pH 5.5 or 7.5, containing 37 KBq ^{59}Fe, 3 μmol/L of iron as ferrous fumarate, electrolytic iron, reduced iron or NaFe-EDTA for 1h at 37°C. Cells were rinsed with PBS-EDTA, trypsinized and counted to measure uptake. Values are means ± SD, n = 3. * Differences statistically significant compared to the same iron compound at pH 5.5, $p < 0.05$.

and counted. Immediately after counting, cells were removed from the gamma counter and kept at 4°C until processing for ferritin concentration.

Determination of ferritin concentration was performed by an ELISA with monoclonal antibodies developed in our laboratory. Cells were homogenized for 60 s in a polytron. The samples and a standard curve were run in duplicate. Also in each plate, duplicate internal controls of known concentration (three different concentrations: low, medium and high) were performed. This ferritin kit has a between assay variation of approximately 7% and a within-assay variation of 5%.

2.4. Statistical analysis.

All measurements were expressed as means ± SD. ANOVA with Bonferroni as post-test was used to compare uptake values and ferritin concentrations for each experiment, except for the study on effect of pH. Paired *t*-test was used for comparisons between the same iron compound at pH 5.5 and 7.5. Values were considered significantly different if $p < 0.05$.

3. Results

3.1. a) Iron uptake from different iron compounds. Effect of ascorbic acid.

Iron uptake from NaFe-EDTA was significantly lower than uptake from other iron sources tested (Fig. 1). While uptake from ferrous fumarate, Ferrochel or electrolytic iron was approximately 54 pmol Fe/mg cell protein (27% of the doses), uptake from NaFe-EDTA was only 10 pmol Fe/mg cell protein (5% uptake).

When ascorbic acid was added, iron uptake increased significantly for all the iron compounds tested, reaching values between 85 and 109 pmol Fe/mg cell protein, which constitutes approximately 50% of the iron administered. In the presence of ascorbic acid, iron uptake from NaFe-EDTA also increased significantly and was not different from the uptake obtained with ferrous fumarate, Ferrochel or electrolytic iron.

3.2. b) Effect of pH.

Due to the low iron uptake obtained from NaFe-EDTA at pH 5.5, experiments were performed increasing the pH of the incubation media to 7.5. Iron uptake at pH 5.5 was 62.02 ± 14.9 pmol Fe/mg cell protein for ferrous fumarate, 63.41 ± 9.1 pmol Fe/mg for electrolytic iron, 60.32 ± 13.7 pmol Fe/mg for reduced iron and 9.17 ± 0.8 pmol Fe/mg for NaFe-EDTA. When the pH of the incubation media was raised to 7.5, iron uptake was significantly reduced for all the compounds tested **(**Fig. 2). Uptake was 1.78 ± 0.6, 2.0 ± 0.4, 2.0 ± 0.8 and 2.6 ± 0.4 pmol Fe/L, for ferrous fumarate, electrolytic iron, reduced iron and NaFe-EDTA, respectively.

3.3. c) Iron uptake from increasing doses of NaFe-EDTA. Effect on uptake from electrolytic iron.

Iron uptake by Caco-2 cells from NaFe-EDTA at 1.5, 3.0, 6.0 and 9.0 μmol/L, was 10.26 ± 2.44, 9.70 ± 3.47, 12.89 ± 3.13 and 13.13 ± 3.23 pmol Fe/mg cell protein, respectively **(**Fig. 3). To each of the NaFe-EDTA concentrations tested, 3μmol Fe/L as electrolytic iron were added

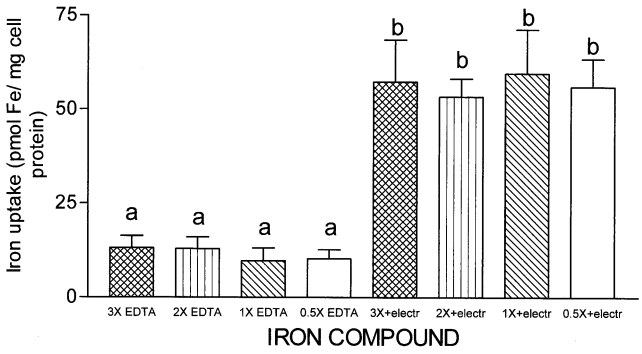

Fig. 3. Effect of increasing doses of NaFe-EDTA and simultaneous addition of electrolytic iron on iron uptake by Caco-2 cells. Cells were incubated with increasing concentrations of NaFe-EDTA at 1.5, 3.0, 6.0 and 9.0 μmol/L. Other iron source, electrolytic iron at 3 μmol/L, was added to the incubation media containing NaFe-EDTA. After 1h incubation at 37°C, cells were rinsed with PBS-EDTA, trypsinized and counted to measure uptake. Values are means ± SD, n = 3. Means with no common letters differ $p < 0.05$.

and uptake increased significantly to 56.06 ± 7.39, 59.60 ± 11.72, 53.39 ± 4.82 and 57.33 ± 11.25 pmol Fe/mg cell protein, respectively. Increases in iron uptake were statistically significant comparing electrolytic iron addition to NaFe-EDTA alone, but there was no difference between electrolytic iron alone (55.29 pmol Fe/mg cell protein, data not shown) and electrolytic iron with NaFe-EDTA at any of the concentrations of NaFe-EDTA tested.

3.4. d) EDTA.

Table 1 shows uptake results from ^{59}Fe alone, electrolytic iron and three different EDTA compounds: NaFe-EDTA, Na_2 -EDTA and K_2 -EDTA. Iron uptake was significantly higher from electrolytic iron than from any of the compounds tested. Uptake from NaFe-EDTA was not different from ^{59}Fe and differences between electrolytic iron and NaFe-EDTA were, as in previous experiments, highly significant.

Iron uptake from Na_2 and K_2 -EDTA was negligible (less than 1 pmol Fe/mg cell protein, and although values were considerably lower than from ^{59}Fe alone, differences resulted statistically no significant. On the other hand, uptake was significantly lower form Na_2 or K_2-EDTA than from NaFe-EDTA or electrolytic iron.

When electrolytic iron was added to incubation media containing NaFe-EDTA, iron uptake did not increase significantly compared to electrolytic iron alone. However in presence of Na_2-EDTA or K_2-EDTA, iron uptake from electrolytic iron was significantly inhibited being K_2-EDTA more potent in decreasing iron uptake. Differences in uptake between Na_2-EDTA or K_2-EDTA were statistically significant.

Table 1
Iron uptake from EDTA and the effect of different forms of this compound on uptake from electrolytic iron by Caco-2 cells[1]

IRON COMPOUND	UPTAKE (pmol Fe/mg cell protein)
^{59}Fe	5.52 ± 2.57^{B,C}
ELECTROLYTIC	68.48 ± 23.0^{A}
NaFeEDTA	10.30 ± 2.22^{B}
Na_2EDTA	0.61 ± 0.20^{C}
K_2EDTA	0.61 ± 0.20^{C}
ELECTROLYTIC + NaFeEDTA	92.67 ± 0.20^{A}
ELECTROLYTIC + Na_2EDTA	10.70 ± 6.26^{B}
ELECTROLYTIC + K_2EDTA	1.96 ± 0.40^{C}

[1] Values are means ± SD. Means with no common letters differ, $p < 0.05$ (n = 4).

3.1. Effect of different iron compounds on uptake and cellular ferritin concentration 24 h post-incubation

Caco-2 cells kept in the incubator with PBS for 24h after the initial 1h incubation with the iron compounds tested (ferrous fumarate, electrolytic iron, reduced iron and NaFe-EDTA), did not show any further change in iron uptake compared to cells processed and counted immediately after 1h incubation. Iron uptake from NaFe-EDTA was significantly lower than uptake from ferrous fumarate, electrolytic iron or reduced iron and similar to uptake from ^{59}Fe alone (Table 2).

Cellular ferritin concentrations were similar for all compounds tested, including ^{59}Fe alone, when cells were ana-

Table 2
Effect of ferrous fumarate, electrolytic iron, reduced iron and NaFe-EDTA on ferritin synthesis by Caco-2 cells[1,2]

Iron compound	Iron uptake (pmol Fe/mg cell protein)	Cellular ferritin concentration (μg/L)	
	1 h	1 h	24 h
^{59}Fe	3.92 ± 0.6^{B}	5.50 ± 0.7^{b}	3.50 ± 2.1^{b}
Ferrous fumarate	61.96 ± 6.5^{A}	1.67 ± 0.6^{b}	20.17 ± 2.8^{a}
Electrolytic iron	55.52 ± 18.5^{A}	1.00 ± 0.1^{b}	26.85 ± 6.5^{a}
Reduced iron	47.74 ± 18.4^{A}	1.00 ± 0.7^{b}	17.50 ± 6.1^{a}
NaFe-EDTA	7.37 ± 1.0^{B}	1.25 ± 0.5^{b}	2.89 ± 1.1^{b}

[1] Ferritin concentration was determined immediately after the 1h incubation period or 24h post incubation, as described in material and methods.
[2] Values are means ± SD. For iron uptake, means in the same column with no common letters differ, $p < 0.05$ (n = 5). For ferritin concentrations comparisons were made for the same column, between different iron compounds and between the same compound after 1 or 24h incubation.

lyzed for ferritin after 1h incubation. However when cells were kept in PBS at 37°C for 24h after 1h incubation, ferritin concentration did change depending on the iron compound. Ferrous fumarate, electrolytic iron and reduced iron produced significant increases compared to 1h incubation. On the other hand, NaFe-EDTA did not produce significant increases in ferritin concentration compared to both, 1h incubation and ^{59}Fe alone. Also, iron uptake from this compound after 24 h in the incubator, was significantly lower than uptake from ferrous fumarate, electrolytic or reduced iron, under the same experimental conditions.

4. Discussion

The high bioavailability of iron from NaFe-EDTA has been repeatedly reported, showing that iron absorption from this compound is at least 2 times higher than absorption from $FeSO_4$, depending on the meal [2,9,10,16,31,32]. Iron uptake results from NaFe-EDTA by Caco-2 cells were unexpectedly low, since this model has been extensively validated regarding iron uptake. This effect does not seem to be due to experimental conditions such as optimal pH since, as a result of the low uptake obtained from NaFe-EDTA, when the pH of the incubation media (usually at 5.5) was increased to 7.5, iron uptake from NaFe-EDTA and from the other 3 compounds tested, decreased significantly.

The enhancing effect of ascorbic acid on iron uptake was confirmed for NaFe-EDTA, resulting in uptake values similar to ferrous fumarate, electrolytic iron and Ferrochel, although without ascorbic acid the uptake from NaFe-EDTA was very low compared to the other 3 iron sources. It has been reported that the use of NaFe-EDTA does not represent any additional benefit over other iron compounds in meals of high bioavailability [1]. Iron absorption studies in humans [10], show that ascorbic acid enhanced iron absorption from a maize porridge when a 1:6 iron:ascorbic acid molar ratio was used. We found that a 1:2 iron:ascorbic acid molar ratio, significantly enhanced iron uptake by Caco-2 cells.

Changes in NaFe-EDTA concentrations above and below 3 mmol/L, which is the concentration usually added for uptake experiments (corresponding to 25X the iron concentration of the isotope), did not produce significant changes in uptake. These results differ from previous reports [22,24, 33,34] showing that iron uptake from Caco-2 cells increases as a function of iron concentration in the incubation media and that there is at least one saturable component. At the concentrations used in this work this saturation state was not reached, since cells were capable of taking iron from other sources.

Addition of electrolytic iron to incubation media containing different concentrations of NaFe-EDTA significantly increased uptake, but the values were similar to the ones obtained with electrolytic iron alone and between the different concentrations of NaFe-EDTA tested. These results, again, did not agree with data published in humans [35] showing that simultaneous addition of NaFe-EDTA and $FeSO_4$ to a corn porridge significantly improved absorption from $FeSO_4$, although there are important differences between both studies such as the compound tested (electrolytic iron is an iron source of low availability) and the fact that for human studies the meal administered contained phytic acid.

Studies with Na_2-EDTA and K_2-EDTA showed an inhibition of iron uptake by Caco-2 cells. When both compounds were administered alone, uptake values were lower (although statistically no significant) than the values obtained from flasks containing only ^{59}Fe, where there was no added iron source. When iron, as electrolytic iron, was added to incubation media containing NaFe- K_2- or Na_2-EDTA, iron uptake from electrolytic iron did not change in presence of NaFe-EDTA while it was significantly inhibited in presence of K_2- or Na_2-EDTA, being K_2-EDTA significantly more potent inhibiting iron uptake. Results obtained in humans demonstrated that the use of Na_2- or Ca_2-EDTA, along with an iron source such as ferrous sulfate, produce high absorption values [16,36].

Iron uptake and ferritin concentration from ^{59}Fe (without added cold iron) was similar to NaFe-EDTA, while ferrous fumarate, electrolytic iron and reduced iron showed a significant increase in uptake after 1h incubation and in ferritin concentration 24h after 1h incubation. Ferritin determinations to Caco-2 cells confirmed uptake values for NaFe-EDTA, since as a result of iron not entering cells, ferritin synthesis did not increase. The increase in ferritin synthesis by Caco-2 cells 24h after 1h incubation with iron was described previously, showing that highly available forms of iron induce greater ferritin formation than less available forms [37].

Besides the unexpected results from EDTA, one interesting issue observed with this model is the consistently similar uptake results from electrolytic iron, and to a lesser extent from reduced iron, compared to highly available forms of iron such as ferrous fumarate or ferrochel. Iron from ferrous fumarate completely enters and exchanges with the non-heme iron pool while for electrolytic or reduced irons, due to their partial insolubility even in acidic solutions, only the soluble fraction enters the pool.

It is also important to highlight that although not statistically significant, differences in ferritin concentrations after incubation with electrolytic or reduced iron were considerable (26.85 mg/L for electrolytic iron and 17.50 mg/L for reduced iron), being uptake and ferritin concentration from electrolytic iron more alike to ferrous fumarate than to reduced iron.

The use of Caco-2 cells as a model to study many human functions, including iron metabolism, has been extensively validated. The system responds to different iron compounds and is susceptible to inhibitors and enhancers of human iron absorption. The low iron uptake values achieved with NaFe-EDTA and the inhibitory effect of Na_2-EDTA by Caco-2

cells is intriguing. Perhaps experimental conditions should be different for this particular compound or maybe there is an intermediate required for uptake that is not present in Caco-2 cells.

It is possible that under in vitro conditions NaFe-EDTA was unable to release iron or give it to an "intermediate", resulting in the low uptake and ferritin concentration values reported. It has been described in humans that EDTA does not enter the enterocyte but it releases iron to apical border [10,18].

From the results of this work it seems evident that the mechanisms or the requirements of iron uptake from EDTA are different than from other iron compounds. These findings require further investigation not only regarding the Caco-2 cells model system, but also re-evaluating the causes of the excellent absorption results reported in humans. It is necessary to evaluate incorporation of iron from NaFe-EDTA to a common pool and the requirements for iron uptake by Caco-2 cells.

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