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In Vitro Study to determine the effect of zinc on non-heme iron absorption

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ABSTRACT
It has been well documented that bioavailability of iron is influenced by other food constituents. We carried out a kinetic study to look at the effect of zinc on ferric iron uptake in human epithelial Caco-2 TC7 cell-line. Zinc reduced iron uptake in a concentration-dependent manner. Iron uptake was significantly increased in the presence of ascorbic acid in the medium. We also found that zinc significantly increased the main iron transporter Divalent Metal Transporter-1 (DMT1) expression in whole cell and membrane proteins. The evidence from in vitro studies show that zinc suppressed iron uptake by Caco-2 cells and this phenomenon could be reversed by the presence of ascorbic acid in the growth media.

Keywords: Iron uptake; Caco-2 TC& cells; Zinc; Ascorbic acid; Ferric iron

Introduction
Iron deficiency is often accompanied by insufficient dietary intake of other micronutrients, especially zinc and copper. This is prevalent in most developing countries because of the low consumption of meat and fortified foods. The diets of infants and children, especially during the weaning period, do not contain adequate amounts of meats, which are good sources of iron and zinc. Thus, deficiencies of both iron and zinc developed, over time. Both zinc and iron are essential micronutrients required for a number of physiological and biochemical functions in the body. The body’s total zinc content ranges from about 1.5 g in women to 2.5 g in men. Just like iron, zinc is an essential component of a large number of enzymes and plays a central role in the immune system. However, zinc can be excreted from the body through the intestines and urine by desquamation of epithelial cells, and in sweat. In the conventional sense, unlike iron, the body has no zinc stores. However, in the case of bone resorption and tissue catabolism, zinc is released and may be reutilized to some extent. The strategy to improve the iron and zinc status of a population by giving combined supplementation of both micronutrients raises the concern of the interactions between these minerals. Although supplements containing iron and multiple trace elements and minerals are used by millions of people worldwide, it is widely recognized that there are interactions between these minerals in the intestinal lumen, affecting their dietary bioavailability. Several studies have reported direct interference reaction in that zinc absorption may be inhibited by high iron concentration and vice versa. For example, when given to adults in solution at a ratio of > 2:1, inorganic iron was found to compete for absorption with zinc. A study conducted by Wasantwisut and co-workers found that when Thai infants aged 4–6 months were given daily supplements of
iron alone for 6 months, hemoglobin and ferritin concentrations were raised which was more than when they were given a combination of iron and zinc. The prevalence of anemia was found to be significantly lower in infants receiving iron only than in infants receiving both iron and zinc.

The uptake of dietary non-haem iron in the gastrointestinal (GI) tract requires protein-mediated transport and two separate enterocyte pathways have been identified, namely the Divalent Metal Transporter-1 (DMT-1) and the Integrin-Mobilferrin pathway (IMP).8 DMT1 requires iron in the ferrous form (Fe^{2+}). As the major proportion of dietary iron is in the ferric form (Fe^{3+}) it needs to be reduced prior to uptake by DMT1 in the enterocytes. Several mechanisms are available to reduce non-heme iron into the ferrous form. The brush border of duodenal enterocytes and cultured intestinal cells possess ferric reductase enzyme activity (Dcytb).9 Following reduction, the Fe^{2+} becomes a substrate for the divalent metal transporter DMT1.10 Iron may also be reduced by dietary factors, the most important being ascorbic acid that can reduce ferric iron (Fe^{3+}) to the more water-soluble ferrous form (Fe^{2+}).11 In contrast to DMT1, the IMP pathway transports no other metals of nutritional importance, only ferric iron (Fe^{3+}), and requires the chelation of Fe^{3+} by mucins before interaction with beta3-integrin and mobilferrin.10,12

Despite extensive study of iron availability, the exact mechanism of iron uptake remains unknown. In this in vitro study, we investigated the influence of zinc on iron uptake and its effect on the expression of iron transporter DMT1 using the in vitro Caco-2 TC7 cell model.

Materials and Methods

Cell culture

Caco-2 TC7 cells were maintained in 25 cm² plastic flasks. The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO, Grand Island, NY USA) supplemented with 20% v/v foetal calf serum (GIBCO), 1% v/v non-essential amino acids (GIBCO), 1% antibiotic antifungal (penicillin-streptomycin) solution (GIBCO) and 1% v/v L-glutamine (Biowhittaker Europe, Verviers Belgium). The cells were maintained at 37°C in an incubator with 5% carbon dioxide (CO2), 95% air atmosphere at constant 95% relative humidity, with the medium replaced every 2 days. For iron uptake experiments, the cells were seeded at a density of 40,000 cells/cm2 in collagen-treated six-well plates (Costar Corp., Cambridge, MA USA) and used at 21 days after seeding.13

Iron Uptake Studies

Caco-2 cells were transferred to serum-free medium 24 hours before the start of experiments. The cells were washed by incubation with buffer (140 mM NaCl, 5 mM KCl, 1 mM NaH2PO4, 10 mM MES, 0.5 mM of MgCl2, 1 mM CaCl2 pH 6.0) for 2 minutes at 37°C. In order to measure iron uptake, 60kBq of 55Fe was added to each culture well and incubated for 15 minutes at 37°C. Uptake was stopped by washing the cells three times with ice-cold buffer and the cells were solubilised in 1 ml of 200 mM NaOH overnight (4°C). Intracellular radioactivity was determined by scintillation counting. The data was fitted to a hyperbolic equation by non-linear regression (SigmaPlot, Systat Software Inc. San Jose, USA):

\[ \text{Uptake} = \frac{V_{\text{max}} \times [\text{FeCl}_3]}{(K_m + [\text{FeCl}_3])} \]

Where \([\text{FeCl}_3]\) is the concentration of substrate, \(V_{\text{max}}\) is the calculated maximum velocity of the reaction and \(K_m\) is the calculated Michaelis constant, an indicator of substrate affinity.14

Cell viability assay
A MTT (3-(4, 5-Dimethythiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay was used to determine cell viability. Briefly, 10µl MTT reagent was added to each well of a 96-well culture plate and incubated for 3-4 hours, at 37°C, before the addition of 100 µl DMSO per well to solubilise the cells. The absorbance of the formazan product was determined at 570 nm. The advantages of the MTT procedure are accuracy, reliability and rapidity in determining cell viability.15

**Ferric Reduction Assay**

The conversion of Fe³⁺ to Fe²⁺ in the absence or presence of ascorbate was measured spectrophotometrically with a chromogenic ferrous iron chelator (bathophenanthrolinedisulfonic acid, BPDS).16 Uptake buffer (700 µl) containing various concentrations of Fe³⁺ (0-30 µM) was added to each cell culture well containing 21-day post-confluence Caco-2 cultures and incubated for 15 minutes at 37°C. The buffer was collected at 4°C in a cold room and 500 µl of 2 mM BPDS added before incubation for 1 hour at 37°C. The concentration of Fe(II)-BPDS complex was measured at 534 nm, using a FeCl₂ standard curve.

**Western Blotting analysis of DMT1 protein levels**

The culture medium was removed and the cell monolayers were subsequently washed twice in phosphate-buffered saline buffer before being harvested with a cell scraper.7 Total plasma membranes (apical and basolateral pooled) were prepared, as described by Mahraoui and co-workers,17 20µg in sample buffer (2M DTT, 250mM Tris, 10% SDS, 50% Glycerol and 0.5% Bromophenol blue), loaded onto a 10% SDS-PAGE and run at 25mA for 45 minutes. Proteins were transferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) and incubated sequentially with a rabbit polyclonal antibody (1:250 dilution) against a synthetic peptide corresponding to amino acids 310-330 of the human DMT1 sequence and a secondary anti-IgG antibody labelled with horseradish peroxidise (HRP). Cross-reactivity was visualised using ECL Plus and Hyperfilm ECL (Amersham Pharmacia Biotech) according to the manufacturer’s instructions, and band densities, corresponding to DMT1, were semi-quantified by densitometric analysis using Scion Image software (Scion Corporation, MD, USA).7

**Statistics and Data Analysis**

Kinetic parameters were determined by fitting the data to a hyperbolic curve by non-linear regression (Prism 5 for Windows, GraphPad Software Inc.). Student t-test was used to determine statistical significance (Prism 5 for Windows, GraphPad Software Inc.).

**Results**

**The Influence of FeCl₃ Concentration on Iron Uptake in Caco-2 Cells**

Caco-2 cells TC7 were treated with different concentrations of Fe(III) before a test dose of radioactive iron was added at appropriate intervals. Fig. 1A shows the uptake of iron in response to increasing concentrations of FeCl₃. In the absence of added dietary components, iron uptake under this condition was maximal at 50pmol. However, all subsequent experiments were performed using 10µM FeCl₃ as this is within the normal range of iron status in the human body.18 Approximately 50% of the iron was taken up by the cells at 10µM FeCl₃ (Fig. 1B).

**Effects of ZnCl₂ on Caco-2 Cell Viability in Serum Free Media and Uptake Buffer**

There was no significant difference between the control and ZnCl₂ treatment on cell
viability (p>0.05) in both mediums. Less than 15% cell death was observed.

**Effect of Increasing Intracellular Zinc Concentration over Iron Uptake in Caco-2 cells TC7**

When intracellular zinc concentration was increased in the buffer, iron uptake decreased in the cells (Fig. 2). A ratio of 1:100 of Fe:Zn inhibited more than 80% of iron uptake.

**Investigating the Kinetic Mechanism on Iron Uptake in Caco2 Cells by adding Zinc into Uptake Buffer**

The purpose of this experiment was to investigate the effect of added zinc and ascorbic acid on iron uptake in Caco-2 cells TC7. The concentration of zinc used in this study ranged from 100μM up to 1000μM and the concentration of ferric iron ranged from 2μM to 30μM. As shown in Fig 3, Caco-2 cells attached to transwell accumulated 200 pmol Fe/well during a 1 hour incubation period in a medium containing 30μM ferric iron without zinc. Zinc reduced iron uptake in a dose-dependent manner. Cellular uptake of iron was reduced by 50% (p < 0.05) when 100μM zinc was added to the uptake buffer.

To examine whether the decrease in cellular acquisition of iron uptake from an uptake buffer containing both iron and zinc followed a Michaelis Menten equation, the levels of Vmax values and Km values were calculated (Table 1). The maximal speed of activity of Fe^{3+} to enter the cells known as Vmax and Km is the concentration of Fe^{3+} required to produce 50% of the Vmax value. Specifically, the analysis of the Km value of iron uptake at different zinc concentrations should allow us to differentiate the modes of inhibition.

To determine whether the decreased uptake of iron from solutions with zinc was associated with a change in the ratio of Fe(III) to Fe(II), 10μM ascorbic acid was added to the uptake solution containing different concentrations of zinc and iron. In the presence of 10μM ascorbic acid, the maximal iron uptake in Caco-2 TC7 cells occurred when the solution contained 100μM zinc. Iron uptake in Caco-2 TC7 cells was found to decrease with further increase in ratio of zinc in the buffer (Fig 4). To examine whether the increase in cellular acquisition of iron uptake from an uptake buffer containing both iron and zinc in the presence of ascorbic acid followed a Michaelis Menten equation, the levels of Vmax values and Km values were calculated. In the presence of ascorbic acid, Vmax values almost 10-fold higher were achieved than in the absence of ascorbic acid (Table 2). However, a similar Km values found for both experiments indicates that zinc actions on iron uptake are a non-competitive inhibition. If it is non-competitive inhibition, increasing the concentration of iron still does not allow the maximum Vmax value to be reached. It means that reduction in Vmax values were not followed by changing Km values.

**Effect of Zinc on DMT1 Expression**

Zinc significantly increased DMT1 levels for both whole cells and membranes (p<0.05) (Fig. 5).

**Discussion**

The body maintains iron homeostasis principally by inversely regulating iron absorption relative to liver iron stores. However, adverse effects of zinc supplementation on iron status have been demonstrated, such as decreased iron absorption, haemoglobin, and serum ferritin levels in adults. Interaction between iron and zinc have been demonstrated in animal experiments and in zinc absorption studies in human volunteers and the physiological basis is the competition of these chemically similar ions for some portions of a common absorptive pathway shared between inorganic (nonheme) iron and zinc. To further
explore the interaction between iron and zinc, we investigated the inhibitory effect of zinc on iron uptake into Caco-2 cells, both in the absence and presence of ascorbic acid. This effect was found to be a non-competitive inhibition. The non-competitive binding means zinc is binding on another transporter spatially distinct from the active site regardless of the presence of iron. Zinc is not competing with iron on the same transporter.23

As there is an unidentified transporter for iron and zinc in Caco-2 cells that is not DMT1 and DMT1 is specific for ferrous iron,24 our findings would imply that DMT1 is not involved in the interaction between iron and zinc as well. Thus, a different transporter system must be involved in the uptake of the iron in the presence of ascorbic acid in the growth media. One possibility is that iron and ascorbic acid forms a complex that interacts in a manner similar to that of Fe³⁺-mucin transport complex,10 while other options include Zip14, a transporter of both iron and zinc in the duodenum, and the SVCT1 ascorbate transporter.25

Western Blotting analysis was done to determine whether differences in the uptake of zinc in Caco-2 cells could have affected the expression of iron transporters (DMT1), western blotting analysis was done. The effect of zinc on DMT1 was intriguing. We found that zinc significantly increased DMT1 expression in whole cell and membrane proteins. New evidence suggested that zinc can indirectly interact with DMT1 by binding and inducing a proton conductance through the transporter.26 Increasing zinc concentration will probably deplete the cells of iron and will indirectly, increase expression of DMT1. This finding did support the previous experiment on iron uptake, which showed that the effect of zinc on iron uptake is non-competitive, implying zinc and iron use different active sites on DMT1 when taken up by the cells. This activity will be impeded when saturated with either iron or zinc in the cells. Several reports suggest that the zinc transporter is distinct from the iron transporter, but the 5’ promoter region of DMT1 contains several metal responsive element consensus sequences which are similar to the promoter of the metallothionein II₄ gene,7,24,27 and are implicated in increased metallothioneine production upon exposure to high zinc levels.7 If zinc was transported the same way as iron, there should be a decline in DMT1 expression. The reason zinc reacts this way is not fully understood. Niles and co workers,18 conducted a study to examine the effect of zinc deficiency on tissue iron concentrations by using Swiss 3T3 cells and concluded that zinc deficiency can result in alterations in iron transporter, storage, and regulatory proteins, which facilitate iron accumulation. Our study, however, did not manipulate the DMT1 pathway directly. Either a knockout or overexpression of the transporter would provide more direct evidence on whether zinc enters the cell via DMT1 or competes with iron for absorption.29

Zinc is critical for the functional and structural integrity of cells. In this work, a zinc-supplemented medium containing either an uptake buffer or serum-free media up to 1000 µM Zn²⁺ was found to be a non-toxic dose for Caco-2 TC7 cells (data not shown). Although at high concentrations zinc can be toxic for pancreatic islet cells,30 and can cause a significant reduction in cell viability as early as four hours in neuroblastoma cells,31 the high zinc content in the experimental buffer has no significant effect on Caco-2 TC7 cells used in the current study. A study conducted by Vega-Robledo and co-workers,31 on the effects of zinc in different cell lines (U-937, human monocytes, and murine bone marrow cells) showed that zinc concentration up to 0.1mM has no effect on the viability of all cells and was similar to that of the controls without zinc.
Conclusion

This study showed that zinc inhibition on iron uptake by Caco-2 TC7 cells occurred when the Fe:Zn ratio was 1:100. Zinc is a non-competitive inhibitor of iron uptake. By adding ascorbic acid, iron uptake was significantly increased in the medium. There was a significant increase in expression of both whole cells and membrane DMT1 levels. Zinc influenced the expression of DMT1. This mechanism could be related to metal responsive elements in the promoter regions of these two key iron transporters in Caco-2 TC7 cells.

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Author Disclosures

The authors have no conflict of interest in the writing of this manuscript.

Biographical notes: Aswir A. Rashed, is a Research Officer at the Nutrition Unit, Institute for Medical Research, Kuala Lumpur. He has participated in several research works at national and regional levels. Currently, he is pursuing his PhD in United Kingdom. His research interests include micronutrients, gut transporters, and functional foods. He serves as a Sub-committee member for Codex Working Group on Food Labelling, Ministry of Health, Malaysia and Sub-committee member for Codex on nutrition and foods for special uses, Ministry of Health, Malaysia. He has several papers published in international journals.

References


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Figure Legends:

**Fig. 1A: Iron uptake (pmol) versus FeCl₃ concentration.** Cells were treated with Fe (III) and exposed to $^{55}$FeCl₃ for 15 minutes, just prior to the uptake study. Iron taken into the cell was measured by scintillation counting. Values are given as pmol/h/well (mean ± sem) for four determinations.
Fig. 1B: The basal rate of iron uptake from 0 -10µM FeCl₃. Cells were treated with Fe (III) and exposed to ⁵⁵FeCl₃ for 15 minutes, just prior to the uptake study. Iron taken into the cell was measured by scintillation counting. Values are given as pmol/h/well (mean ± sem) for four determinations.
Fig. 2: Competition studies between Fe and Zn in Caco-2 TC7 cells. Cells were incubated with different concentrations of ZnCl₂ and FeCl₃ exposed to \(^{55}\)FeCl₃ for 15 minutes just prior to the uptake study. Iron taken into the cell was measured by scintillation counting. Iron uptake was measured after removing loosely bound \(^{55}\)Fe from the cell surface by multiple washings with ice-cold buffer. Values are given as pmol (mean ± sem) for four determinations.
Fig. 3: **Zinc inhibits iron uptake in Caco-2 TC7 cells.** Cells were incubated with different concentrations of ZnCl₂ and FeCl₃ exposed to $^{55}$FeCl₃ for 15 minutes just prior to the uptake study. Iron taken into the cell was measured by scintillation counting. Iron uptake was measured after removing loosely bound $^{55}$Fe from the cell surface by multiple washings with ice-cold buffer. Values are given as pmol (mean ± sem) for four determinations. Michaelis-Menten equation: $Y = (V_{max} \times X)/(K_m + X)$. 

![Graph showing the inhibition of iron uptake by zinc](image-url)
Fig. 4: Ascorbic acid eliminates the effect of zinc on iron uptake in Caco-2 TC7 cells. Cells were incubated with different concentrations of ZnCl₂, 10µM ascorbic acid and FeCl₃ exposed to ⁵⁵FeCl₃ for 15 minutes just prior to the uptake study. Iron taken into the cell was measured by scintillation counting. Iron uptake was measured after removing loosely bound ⁵⁵Fe from the cell surface by multiple washings with ice-cold buffer. Values are given as pmol (mean ± sem) for four determinations. Michaelis-Menten equation: \( Y = \frac{V_{\text{max}} \times X}{K_m + X} \).
Table 1: Kinetic parameters of the Michaelis-Menten in the absence of ascorbic acid.

<table>
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<th>[Zinc] µM</th>
<th>$V_{\text{max}}$ values (pmol/h)</th>
<th>$K_m$ values</th>
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</tr>
<tr>
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<tr>
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<td>74</td>
<td>11.8</td>
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Table 2: Kinetic Parameters of the Michaelis-Menten in the Presence of Ascorbic Acid

<table>
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<tr>
<th>Ascorbic acid 10µM + [Zinc] µM</th>
<th>$V_{\text{max}}$ values (pmol/h)</th>
<th>$K_m$ values</th>
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<td>100</td>
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<td>1000</td>
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</table>
Fig. 5: The effect of added ZnCl₂ on whole cell and membrane DMT1 levels. Cells were treated for 24 hours with 1000µM ZnCl₂. Protein samples were taken and subject to Western blotting. Band density was measured using Scion image software. Values were expressed as mean ± sem of three experiments. *Significant difference from control p<0.05 (student t-test).