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Inhibition of iron and copper uptake by iron, copper and zinc

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ABSTRACT

Interactions of micronutrients can affect absorption and bioavailability of other nutrients by a number of mechanisms. In aqueous solutions, and at higher uptake levels, competition between elements with similar chemical characteristics and uptake process can take place. The consequences of these interactions may depend on the relative concentrations of the nutrients. In this work, we measure the effects of increasing concentrations of iron, zinc, and copper on iron and copper uptake in Caco-2 cells. Intracellular Fe or Cu levels were affected by incubating with increased concentrations of metals. However, when the cells already had different intracellular metal concentration, the uptake of Fe or Cu was nor affected. In competition studies, we showed that Cu and Zn inhibited Fe uptake, and while Fe inhibited Cu uptake, Zn did not. When the three metals were given together (1: 1: 1 ratio), Fe or Cu uptake was inhibited ~40%. These results point to a potential risk in the absorption and bioavailability of these minerals by the presence of other minerals in the diet. This aspect must be considered in food supplementation and fortification programs.

Key terms: competition, interaction, metals, uptake

INTRODUCTION

Iron, copper and zinc are essential metals for humans. They are required for the metabolic activities of over 70 metalloenzymes. However, due to their redox activity, iron and copper also can be toxic. The intestinal competition of zinc with copper, iron, lead, calcium and cadmium may accentuate nutritional deficiencies or toxicities from these environmental metals (Abdel-Mageed et al., 1990).

Iron deficiency is the single most common nutritional disorder worldwide; approximately 2 billion persons worldwide suffer from iron deficiency (WHO, 1999), and this disorder is the main cause of anemia in infancy, childhood and pregnancy. Iron deficiency is prevalent in most of the developing world and is probably the only nutritional deficiency worthy of consideration in industrialized countries. Iron deficiency is often accompanied by other micronutrient deficiencies such as zinc and copper, especially when caused by an insufficient dietary intake of micronutrients, as is often the case in developing countries (Solomons and Ruz, 1997; Allen, 1994). As a result, supplements containing iron and multiple trace elements and minerals are used by millions of people worldwide. (Troost, 2003). Food fortification and supplementation with several micronutrients are strategies currently utilized to prevent combined micromineral deficiencies. Infant formulas are usually fortified with iron, zinc, copper and ascorbic acid. A unifying hypothesis is not yet established for the effects or imbalances among these elements. These interactions will be of substantial practical importance in

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estimating dietary recommendations, in validating prophylactic measures, and in the assessment of situations in which human and animal health may be at risk. Because of their similar physicochemical properties and shared absorptive pathways, these microminerals may have negative interactions. However, the studies that have analyzed the interactions among iron, zinc, and copper absorption have shown conflicting results. Furthermore, information is scarce about the optimal molar ratios that minimize these potential negative interactions. The aim of the current study was to measure the effects of increasing concentrations of iron, zinc, and copper on iron and copper uptake in Caco-2 cells.

METHODS

Reagents

Iscove's medium was purchased from GIBCO Life Technologies (Carlsbad, CA). Fetal Bovine Serum (FBS) was from Clontech (Palo Alto, CA). ^{64}Cu was from *Comisión Chilena de Energía Nuclear* (Santiago, Chile).

Cell growth

Caco-2 cells were grown in Iscove´s medium + 10% FBS and cultured as described elsewhere (Arredondo et al., 2004). Approximately $1x10^5$ cells were grown in bottles (25 cm^2 growth area) for seven days at 37º C, 5% CO_2. Medium was changed every two days. After seven days, cells were confluent (1.5 to 3.0 x 10^6 cells/ml approximately). Caco-2 cells were incubated with Fe ($FeCl_3 \cdot 6H_2O$) as Fe-NTA (1: 2), Cu ($CuSO_4$) as Cu-histidine (1: 10), and Zn ($ZnSO_4 \cdot 7H_2O$) as $ZnCl_2$. The experiments were performed in the presence of ascorbic acid (1: 10; metal: AA) to maintain metal ions in reduced state (Fe^{2+} and Cu^{1+}).

Pre-treatment of Caco-2 cells with different extracellular metal concentrations. To obtain cells with different intracellular metal concentration, Caco-2 cells were seeded at equilibrium in 12-well plates with Iscove´s medium + 10% FBS plus different concentrations (0 to 100 μM) of Fe-NTA (1: 2), Cu-histidine (1: 10), $ZnCl_2$ or a combination of these. Medium was changed every two days.

Cellular extracts

Cellular extracts were prepared from Caco-2 cells grown as described above. Cells were washed twice with PBS and then incubated with saline-Tris buffer (in μM: 40 Tris-HCl, 100 NaCl, pH 7.5 and 1 EDTA) for 10 minutes at 37ºC. The cell suspensions were transferred to a 1.5 ml micro tube and centrifuged 1 minute at 1000 rpm. The pellet was resuspended in 50 μL lysis buffer (in mM: 10 Hepes pH 7.5, 3 $MgCl_2$, 40 KCl, 1 PMSF, 1 dithiothreitol, and 0.5% NP-40, 10 ug/ml leupeptin, 0.5 ug/ml aprotinin, 0.7 ug/ml pepstatin, 5% glycerol) and incubated 15 minutes at 4º C, then centrifuged 10 minutes at 10,000 g. The supernatant was transferred to a 0.5 ml micro tube then 25 μL saline-Tris buffer added. A 10 μL aliquot was taken for protein determination by using the Lowry method (1951).

Intracellular metal concentrations

To determine Fe and Cu intracellular concentrations, Caco-2 cells were incubated at equilibrium with different concentrations of the single metals (between 0 and 100 μM) for 7 days. The cells were washed and a cellular extract was prepared. Cellular extract was digested with concentrated ultrapure nitric acid (1: 1) overnight at 60ºC. Fe and Cu content were determined by using an atomic absorption spectrometer (AAS) equipped with graphite furnace (SIMAA 6100, Perkin Elmer, Shelton, CT). MR-CCHEN-002 (Venus antiqua) and DOlt-2 (Dogfish liver) preparations were used as reference materials to validate the mineral analyses.

Metal uptake

To determine ^{55}Fe and ^{64}Cu uptake on Caco-2 cells incubated at equilibrium with different concentrations of metal, Caco-2 cells were incubated as described and supplemented with 5, 10, 20 and 50 uM of Fe, Cu or Zn for 7 days. Cells were washed and uptake was performed using 10 uM ^{55}Fe

or ^{64}Cu for different times in transport buffer (In mM: 50 MOPS-Na, 94 NaCl, 7.4 KCl, 0.74 $MgCl_2$, 1.5 $CaCl_2$, 5 glucose, pH 7.4). Cells were washed and intracellular metal (radioisotope and total) concentration was measured in a Beta counter and by AAS.

Competition studies

For competition studies, Caco-2 cells were grown for 7 days as above and then incubated in transport buffer with a) ^{55}Fe: Zn, ^{55}Fe: Cu, ^{64}Cu: Fe or ^{64}Cu: Zn in varying ratios from 1: 0.5 to 1: 200, using 5 uM of ^{55}Fe or ^{64}Cu to other metal (2.5-1000 uM) (results were expressed as a % of baseline); or b) ^{64}Cu: Fe: Zn or ^{55}Fe: Cu: Zn (1: 1: 1, 1: 10: 5). After the incubation, cells were washed and intercellular radioisotope was measured.

RESULTS

Intracellular content of Fe, Cu and Zn in Caco-2 cells

Intracellular content of Fe, Cu and Zn in Caco-2 cells incubated with Fe or Cu are shown in figures 1A and 1B, respectively. As expected, when extracellular Fe increased in the media (0.5 to 100 uM), intracellular Fe increased from 0.051 to 1.914 ug/mg protein (one-way ANOVA, $p<0.001$). However, in the same conditions, both Cu and Zn decreased from 0.100 to 0.041 ($p<0.05$) and 0.381 to 0.041 ug/mg protein ($p<0.01$), respectively (Fig. 1A). When Caco-2 cells were incubated with increasing Cu concentrations, intracellular Cu content increased from 0.065 to 0.744 ug/mg protein (one-way ANOVA, $p<0.001$). In this situation, Fe decreased from 0.161 to 0.029 ($p<0.01$) and Zn had just a marginal decrease, 0.082 to 0.079 ug/mg protein, respectively (Fig. 1B). Cells incubated in basal conditions, had a ratio of 1: 2: 4 of Fe: Cu: Zn, respectively.

Effect of increasing intracellular concentrations of Fe, Cu or Zn over Fe or Cu uptake

To study the effect of the intracellular metal content on the uptake of Fe or Cu, Caco-2 cells were incubated with four concentrations (0.5, 10, 20 and 50 uM) of Fe, Cu or Zn for 7 days. Independently of

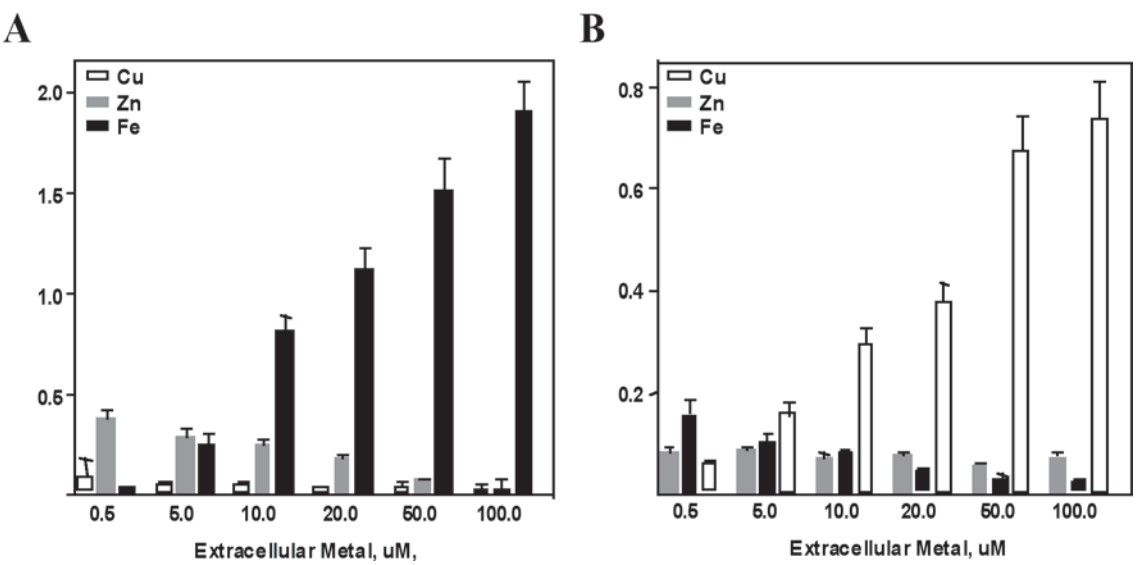

Figure 1: Intracellular Fe or Cu content in Caco-2 cells incubated with different extracellular metal concentrations. Caco-2 cells were incubated with different concentration of Fe, Cu or Zn (0.5 – 100 μM) for 1 week, and the intracellular Fe (A) or Cu (B) concentrations was measured in a cellular extract by EAA with graphite furnace. (n=5 different experiments).

the intracellular Cu or Zn concentrations (Figs. 2A and 2B), ^{55}Fe uptake was not affected. However, Fe uptake in cells grown in different Cu concentrations was lower than in cells grown in different Zn concentrations. In the same way, intracellular Fe or Zn concentrations (Figs. 3A and 3B) do not affect ^{64}Cu uptake. (One-way ANOVA , p = NS). Cu uptake in cells grown in different Zn concentrations was lower than in cells grown in different Fe concentrations.

Studies of competition between metals (Fe vs. Cu or Zn and Cu vs. Fe or Zn)

^{55}Fe uptake decreased when the extracellular concentrations of Cu (Fig. 4, squares) or Zn (Fig. 4, circles) increased. The concentrations of metal ions that inhibited Fe uptake by 50% were 8.1 uM of Cu or 8.3 uM of Zn. ^{64}Cu uptake also decreased when extracellular Fe concentrations increased (Fig. 5, squares). Fe at 11.5 uM inhibited Cu uptake by 50%. However, increased extracellular Zn concentrations did not affect Cu uptake (Fig. 5, circles).

Effect of different molar ratios over ^{55}Fe or ^{64}Cu uptake

Caco-2 cells were incubated with different molar ratios of ^{64}Cu: Fe: Zn or ^{55}Fe: Cu: Zn (1: 1: 1 a 1: 10: 5; from 10: 10: 10 uM to 10: 100: 50 uM), and the uptake of Fe and Cu was studied. Under these conditions, a ratio of 1: 1: 1 of ^{64}Cu: Fe: Zn (Fig. 6A) or ^{55}Fe: Cu: Zn (Fig. 6B) inhibits 40% and 32% of ^{64}Cu and ^{55}Fe uptake, respectively.

DISCUSSION

Zn, Cu and Fe are essential mineral elements that exhibit important interactions and possible competitive inhibition of transport and bioavailability (Reinstein et al. 1984; Brewer et al. 1985). In this work, we studied the effects of iron, copper and zinc, alone or in combination in different metals ratios, on the absorption of each other.

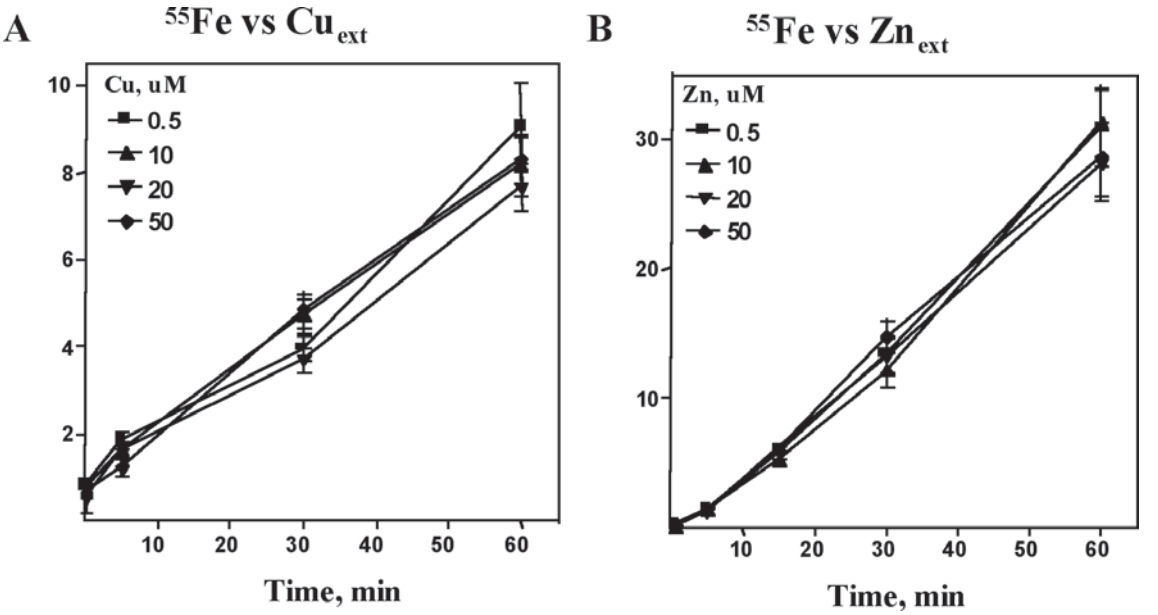

Figure 2: Apical Fe uptake in Caco-2 cells pre-exposed to different extracellular Cu or Zn concentration. Caco-2 cells were incubated for 2 week with different Cu (A) or Zn (B) concentration. On the day of the experiment, cells were incubated with 10 μM of ^{55}Fe. Uptake was stopped with PBS-EDTA. Radioactivity was determined in the cellular extracts and expressed as ^{55}Fe uptake in pmole per mg of protein. (n=3 independent samples).

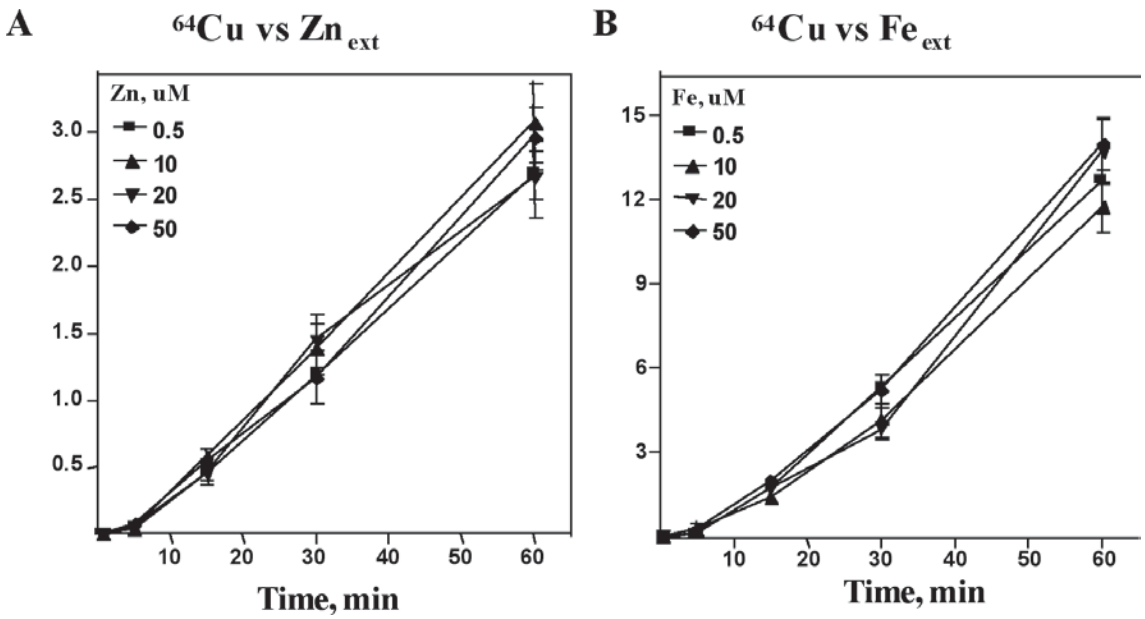

Figure 3: Apical Cu uptake in Caco-2 cells exposed to different extracellular Fe or Zn concentration. Caco-2 cells were incubated for 2 week with different Fe (A) or Zn (B) concentrations. On the day of the experiment, the cells were incubated with 10 μM of ^{64}Cu. Uptake was stopped with PBS-EDTA. Radioactivity was counted in the cellular extracts and expressed as ^{64}Cu uptake in pmole per mg of protein. (n=3 independent samples).

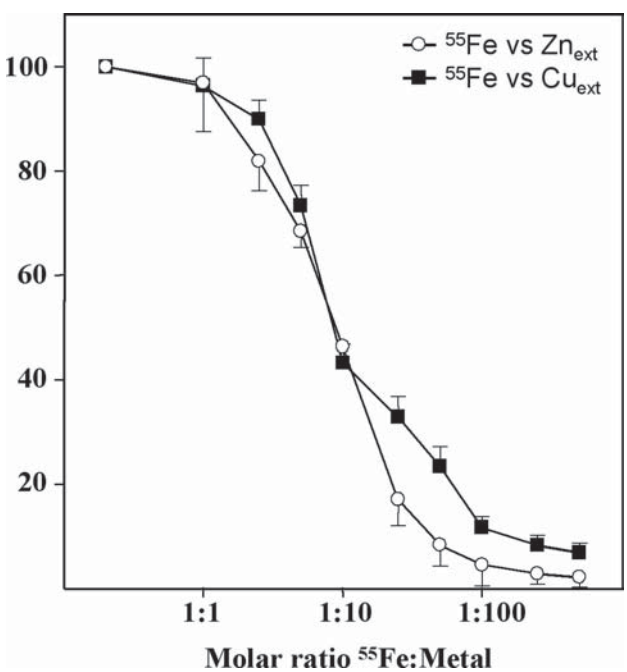

Figure 4: Competition studies between Fe vs. Cu or Zn in Caco-2 cells. Caco-2 cells were grown in regular culture media. On the day of the experiment, the cells were incubated with increased concentrations (2.5 to 1000 μM) of Cu (squares) or Zn (circles) and 5 μM of ^{55}Fe. Uptake was stopped with PBS-EDTA. Radioactivity was counted in the cellular extracts and expressed as percentage of ^{55}Fe uptake relative to baseline uptake defined as a 100% uptake. (n=3 independent samples).

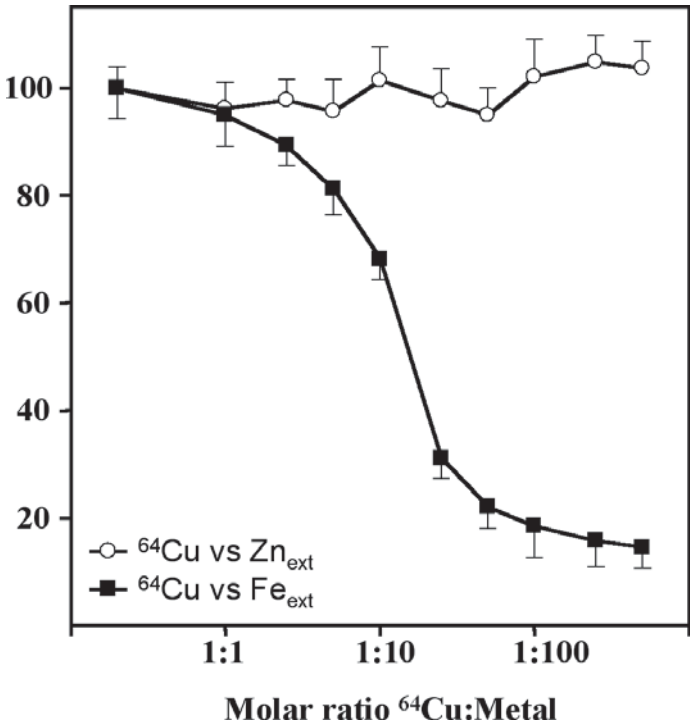

Figure 5: Competition studies between Cu vs. Fe or Zn in Caco-2 cells. Caco-2 cells were grown as above and then incubated with increased concentrations (2.5 to 1000 μM) of Fe (squares) or Zn (circles) and 5 μM of ^{64}Cu. Uptake was stopped with PBS-EDTA. Radioactivity was counted in the cellular extracts and expressed as percentage of ^{64}Cu uptake relative to baseline uptake defined as a 100% uptake. (n=3 independent samples).

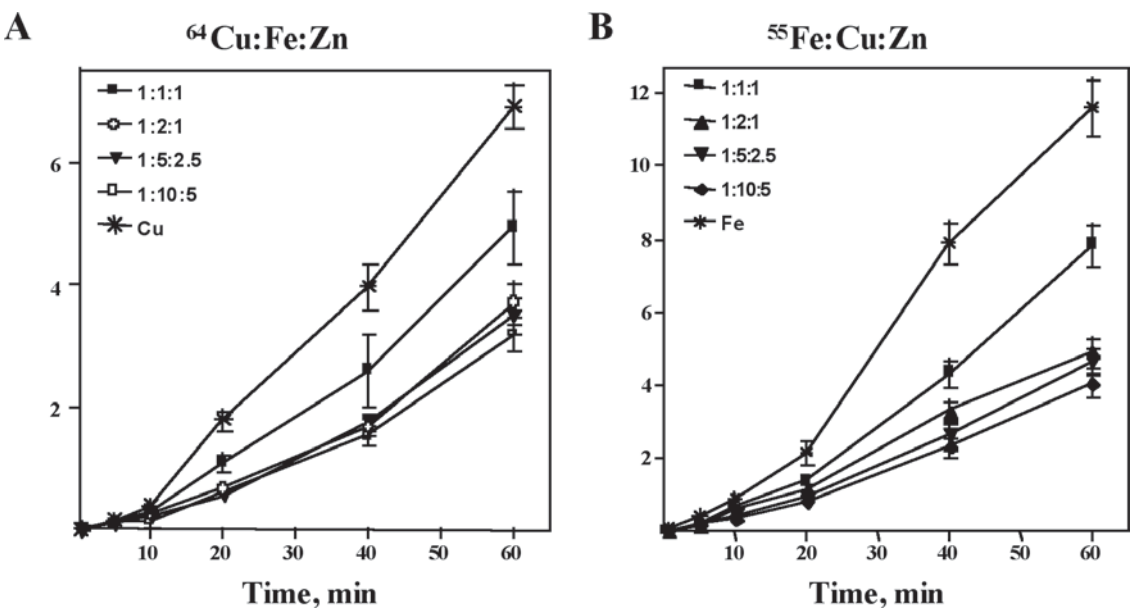

Figure 6: Different molar ratios of Fe, Cu and Zn. Caco-2 cells were incubated with different molar ratios of ^{55}Fe: Cu: Zn (A) or ^{64}Cu: Fe: Zn (B). As a control, ^{55}Fe or ^{64}Cu uptake alone was used. Uptake was stopped as before and radioactivity counted in the cellular extracts and expressed as ^{55}Fe or ^{64}Cu uptake in pmole per mg of protein.. (n=3 independent samples).

We showed that the incubation of Caco-2 cells with Fe, Cu or Zn, affects Fe metabolism. Similar results in Caco-2 and HepG2 cells were reported earlier by us (Arredondo et al., 2000; 2004). As expected, when extracellular iron concentrations increased in the media, intracellular Fe increased as well. However, when Cu or Zn increased in the extracellular media, a decrease in intracellular Fe was observed in both conditions. Similarly, cells incubated with Fe, Cu or Zn showed altered Cu metabolism. When Cu increased in the media, the intracellular Cu also increased. When Fe increased in the media, intracellular Cu decreased. However, when Zn increased, intracellular Cu did not change. These results confirm previous studies in which Fe was shown to decrease zinc absorption in humans in a dose-dependent way when given in a water solution but not when given with a meal (Valberg et al., 1984; Sandstrom 1985) and when oral iron therapy impaired zinc absorption and thus zinc status in a dose-independent fashion, without affecting copper absorption (Troost, 2003).

The uptake of ^{55}Fe did not change in cells that were pre-incubated with different concentrations of Zn or Cu. This means that independently of the intracellular status of metals, when Fe is given alone, absorption is not inhibited. The same pattern was observed for ^{64}Cu uptake when the cells were pre-incubated with Zn or Fe.

In competition studies, we showed that Cu or Zn inhibited Fe uptake and Fe inhibited Cu uptake. However, Zn did not inhibit Cu uptake. When two metals were given together there was an evident inhibition in the uptake, especially over 1: 10 molar ratio. When the three metals were given together in different ratios, a molar ration of 1: 1: 1 (^{64}Cu: Fe: Zn or ^{55}Fe: Cu: Zn) inhibited the uptake of Cu or Fe by ~40%. Based on these results, a concern arises about the concentrations of minerals currently used in the programs of food fortification with multinutrients. These findings are especially relevant for the infantile milk formula field, since most of these preparations are fortified with copper, iron and zinc in molar ratios of 1: 20: 5. We demonstrated a ~60% inhibition of copper uptake with molar ratios of 1: 10: 5. Future research is required to find the optimal molar ratios among these microminerals.

Zn-Cu competition has been exploited beneficially for the treatment of Wilson's disease to avoid Cu accumulation (Barone, 1998). Recent progress in the field of metal ion transport has advanced significantly our understanding of the mechanisms of intestinal metal ion absorption under normal and pathological conditions. Excessive intake of one of these elements, beyond its normal presence in the diet, may result in an overt deficiency of another element. A particular example is the indiscriminate use of high-dose, over-the-counter minerals supplements. The interaction between Zn and Cu can result in various manifestations of Cu deficiency, including microcytic anemia and neutropenia (Botash et al. 1992; Gyorffy and Chan 1992).

The interaction between iron, copper and zinc absorption may be explained by competitive binding to the transporter protein DMT1, which participates in divalent metal transport (Fe, Cu, Zn, Mn, Pb) (Gunshin, 1997). We had demonstrated previously that DMT1 is the main Fe^{2+} transporter, that it participated actively in Cu^{1+} transport (Arredondo, 2003), and that this transporter could be regulated by both Fe and Cu (Arredondo, 2004). Long exposure to iron leads to a down-regulation of DMT1 expression that subsequently will produce a decrease in Cu and Zn absorption (Troost, 2003). Furthermore, recent findings suggest that a shared absorption pathway for iron and zinc is distinct from DMT1, although the actual absorption mechanism remains to be elucidated (Yamaji et al., 2001). The dietary proportions of zinc, copper, and iron appear to influence zinc, copper, and iron metabolism at the intestinal and cellular transport levels over a given period of time (Abdel-Mageed and Ohm, 1991).

In summary, Fe, Cu and Zn affect the uptake of one another in a model of intestinal epithelia. A number of inhibitory interactions between these minerals could take place when high doses of a single

minerals is given or when the supply is a mix of minerals. The potential risk for interactions should be considered in food fortification or supplementation programs.

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