Iron and zinc bioavailability in Caco-2 cells: Influence of caseinophosphopeptides

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ABSTRACT

A study has been made of the influence of two pools of caseinophosphopeptides (CPPs) obtained from αs- and β-casein (CN) fractions, and of three specific CPPs (β-CN(1–25)4P, αs-CN(64–74)4P and αs2-CN(1–19)4P), on iron bioavailability (ferritin synthesis) and zinc bioavailability (retention, transport and uptake of zinc) in Caco-2 cells.

α-CPP and β-CPP pools did not improve ferritin synthesis, but the three specific CPPs showed an increase in ferritin synthesis in Caco-2 cells versus iron sulphate, β-CN(1–25)4P being the most effective. In relation to zinc bioavailability, αs-CPPs, β-CPPs, αs1-CN(64–74)4P and β-CN(1–25)4P increased zinc uptake. However, this increase was of the same order as the increase due to the presence of zinc sulphate.

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1. Introduction

The digestion of milk proteins releases bioactive peptides encoded in the native proteins, which can exert specific physiological functions in the gastrointestinal, immune, cardiovascular and nervous systems (Hartmann & Meisel, 2007). Among them, caseinophosphopeptides (CPPs) have divalent metal ion (e.g., iron and zinc) sequestering activity through a sequence of three phosphoserine, followed by two glutamic acid residues (SpSpSpEE). This property implies that CPPs could be used as supplements for fortifying food, with a view to improving mineral bioavailability (Meisel & Fitzgerald, 2003).

In this sense, the positive influence of β-CN(1–25)4P on iron bioavailability in rats has been described (Ait-Oukhatar et al., 1997, 1999, 2002; Ani-Kibangou et al., 2005; Kibangou et al., 2005a; Pérès et al., 1999a, 1999b). In Caco-2 cells, β-CN(1–25)4P decreased iron transport and increased iron retention and uptake versus iron gluconate (Kibangou et al., 2005a), and increased iron transport (Kibangou et al., 2005b, 2008) and decreased iron retention and uptake (Kibangou et al., 2008) versus iron sulphate. Besides, it was reported that CPPs, proceeding from different fractions of CN (αs- or β-CN), exert different effects on iron bioavailability. In Caco-2 cells, the iron bound to β-CN(1–25)4P showed greater uptake than did iron bound to αs1-CN(59–79)5P (Kibangou et al., 2005b) or to a mixture of αs2-CN(59–79)5P and αs2-CN(2–21)4P (Kibangou et al., 2005a). This observation had been previously reported by Bouhallab et al. (2002) who, in a perfused rat duodenal loop system, observed greater uptake and net absorption of iron bound to CPPs from β-CN, containing β-CN(1–25)4P, versus αs-CN fractions. These results suggest that the structure and conformation of CPPs are important. In a previous study by our group, involving infant formulas subjected to simulated gastrointestinal digestion, we found that iron showed greater binding to released CPPs from β-CN versus αs-CN fractions (Miquel, Alegría, Barberá, Ferré, 2005). This was again confirmed in a subsequent study where the addition of fruit beverages of pools of CPPs obtained from CN subjected to simulated gastrointestinal digestion did not improve iron uptake in Caco-2 cells (García-Nebot, Alegúa, Barberá, Clemente, & Romero, 2010a). This result was attributed to a greater percentage of CPPs proceeding from αs-CN versus β-CN fractions (García-Nebot, Alegúa, Barberá, Contreras, & Recio, 2010b). However, the same pool of CPPs increased ferritin synthesis when it was added to fruit beverages supplemented with iron (García-Nebot et al., 2010c), ferritin synthesis being a more sensitive marker of iron bioavailability.

On the other hand, in infant formulas subjected to simulated gastrointestinal digestion, it was seen that zinc showed greater binding to released CPPs from αs-CN versus β-CN fractions (Miquel et al., 2005). Despite this observation, the addition of pools of CPPs containing a greater percentage of CPPs proceeding from αs-CN versus β-CN fractions did not increase zinc retention, transport or uptake in Caco-2 cells (García-Nebot, Alegúa, Barberá, Clemente, & Romero, 2009). Regarding the effect of CPPs proceeding from different fractions of CN, we know of only one study that has evaluated the influence of specific CPPs on zinc bioavailability (Pérès et al., 1998). These authors reported improvement of zinc absorption using β-CN(1–25)4P versus zinc sulphate in a perfused rat loop model.

Abbreviations: CN, casein; CPPs, caseinophosphopeptides; FBS, fetal bovine serum; MEM, minimum essential medium; PBS, phosphate buffered saline.
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Taking into account the different effects upon iron bioavailability, according to the kind of CPPs, and the few studies of these effects in relation to zinc bioavailability, the present study was designed to compare the influence of two pools of CPPs obtained from \( \alpha_s \) - and \( \beta-CN \) fractions on iron and zinc bioavailability in Caco-2 cells. Furthermore, we assayed the influence upon iron and zinc bioavailability of three specific peptides: \( \beta-CN(1–25)4P \) used as reference CPP due to its known influence upon iron bioavailability, and another two CPPs, \( \alpha_{s1-CN}(64–74)4P \) and \( \alpha_{s2-CN}(1–19)4P \), since a previous study by our group confirmed their resistance to simulated gastrointestinal digestion (García-Nebot et al., 2010b).

2. Materials and methods

2.1. Reagents

2.1.1. Digestion

Pepsin (porcine; Cat. No. P7000), pancreatin (porcine; Cat. No. P1750) and bile extract (porcine; Cat. No. B8631) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The working solutions of these enzymes were prepared immediately before use.

Water of cellular grade (Aqua B Braun, Braun Medical, Barcelona, Spain) or Milli-Q water (18.2 MΩ cm resistivity) was used for the preparation of all reagents. CaCl\(_2\) was purchased from Panreac (Barcelona, Spain).

Standard Zn solutions were prepared immediately before use by dilution with Milli-Q water (18.2 MΩ cm resistivity) of a standard solution of 1000 mg/l (Titrisol, Merck, Barcelona, Spain).

2.1.2. Caco-2 cells

Minimum essential medium (MEM), phosphate buffered saline (PBS), and trypsin–EDTA solution, fetal bovine serum (FBS), nonessential amino acids, \( \beta \)-glutamine, antibiotic solution, and fungizone were from Gibco BRL Life Technologies, Scotland. Transport buffer for the zinc bioavailability assays contained 130 mM NaCl (Merck), 10 mM KCl (Merck), 1 mM MgSO\(_4\) (Sigma Chemical Co.), 5 mM glucose (Sigma Chemical Co.) and 50 mM HEPES (Gibco, Scotland) at pH 7.4.

2.2. Samples

2.2.1. \( \alpha \)-CPP and \( \beta \)-CPP pools

Two pools of CPPs, \( \alpha \)-CPPs and \( \beta \)-CPPs were obtained from \( \alpha_s \) - and \( \beta-CN \) (Sigma Chemical Co., St. Louis, MO, USA), respectively, subjected to simulated gastrointestinal digestion according to the method of García-Nebot et al. (2010b), using an intestinal pH of 6.5. Briefly, both samples were digested using two demineralised enzymatic solutions (pepsin solution in the gastric stage at pH 2 for 2 h at 37 °C, and pancreatin–bile solution in the intestinal stage at pH 6.5 for 2 h at 37 °C), the pH was adjusted to 7.2, and the whole then centrifuged (3500g, 60 min at 4 °C). The CN not digested was precipitated at pH 4.6, and then the supernatants were selectively precipitated with CaCl\(_2\) and absolute ethanol at pH 8. The preparation was then centrifuged at 12,000g for 10 min at 10 °C. The precipitate was washed with 50% (v/v) ethanol, lyophilised, and stored at −20 °C.

The CPPs generated were identified by HPLC–ESI-MS/MS according to the method of García-Nebot et al. (2010b). In the pool of \( \alpha \)-CPPs we identified a total of 16 CPPs of molecular weights between 671 and 2881 Da, of which 9 were derived from \( \alpha_{s1-CN} \), and 7 from \( \alpha_{s2-CN} \), containing 4 phosphopeptides with the cluster (SpSpSpEE), and 24 residues of phosphoserine with a chain length between 5 and 22 amino acids. In the pool of \( \beta \)-CPPs, we identified a total of 18 CPPs of molecular weights between 975 and 2985 Da, containing 4 phosphopeptides with the cluster (SpSpSpEE), and 17 residues of phosphoserine with a chain length between 7 and 23 amino acids.

2.2.2. Specific CPPs

\( \beta-CN(1–25)4P \) was purified from a tryptic hydrolysate of bovine \( \beta-CN \), and two synthetic phosphopeptides (\( \alpha_{s1-CN}(64–74)4P \) and \( \alpha_{s2-CN}(1–19)4P \)) were purchased from Peptide 2.0 (Chantilly, USA).

2.3. Caco-2 cells

2.3.1. General

Caco-2 cells were obtained from the American Type Culture Collection (HTB-38, Rockville, MD, USA), and were used between passages 40 and 55.

For the assays on ferritin synthesis, the cultures were maintained and grown as previously described (Viadel, Perales, Barberá, Lagarda, & Farré, 2007), under low iron conditions. The cells were maintained in 75 cm\(^2\) flasks (IWAKI brand) in MEM supplemented with 10% (v/v) FBS depleted of iron, 1% (v/v) nonessential amino acids, 1% (v/v) \( \beta \)-glutamine, 1% (v/v) antibiotic solution, and 0.1% (v/v) fungizone at pH 7.2–7.4 in an incubator under a 5% CO\(_2\)/95% air atmosphere at constant humidity. For the assays on zinc retention, transport and uptake, the cultures were maintained and grown as previously described for ferritin synthesis, except that, in this case, MEM was supplemented with FBS without iron depletion (García-Nebot et al., 2009). Four independent replicates were performed for each experiment, and each experiment was repeated twice.

2.3.2. Ferritin assay

For these assays, we analysed a total of 11 samples prepared in MEM supplemented with FBS depleted of iron: iron control (50 \( \mu \)M Fe\( \text{SO}_4 \)), CPPs control (pool of \( \alpha \)-CPPs, pool of \( \beta \)-CPPs, \( \alpha_{s1-CN}(64–74)4P \), \( \alpha_{s2-CN}(1–19)4P \) and \( \beta-CN(1–25)4P \)) at 12.5 \( \mu \)M, and these same CPPs in the presence of iron sulphate (50 \( \mu \)M, Fe:CPP molar ratio 4:1) after mixing for 30 min at 37 °C.

The ferritin measurement method was applied as previously described by García-Nebot et al. (2010c). The cells were seeded at a density of 50,000 cells/cm\(^2\) in 6-well plates.

The culture medium was removed from each well, the cell monolayer was washed with PBS at 37 °C, and then 2 ml of the sample solution were added to the cell monolayer. Cell cultures were incubated for 2 h at 37 °C in 5% CO\(_2\) with 95% relative humidity, and then this solution was removed and replaced by culture medium, and the cells were returned to the incubator for an additional 22 h.

Cell monolayers were washed with PBS and detached with trypsin–EDTA solution. Subsequently, cells were collected with 2 ml of water of cellular grade at 4 °C and homogenised at 17,000 rpm for 3 min at 4 °C (Polytron \(^\text{TM}\) PT 2000, Kinematica AG). Ten-millilitre aliquots of the sonicated Caco-2 monolayer were used for ferritin determination (Spectro Ferritin kit, Cat. No. S-22, Ramco Laboratories Inc., Stafford, TX, USA).

2.3.3. Zinc retention, transport and uptake assays

For these assays, we analysed the same samples as in the ferritin assays, using zinc (Fe\( \text{SO}_4 \), 50 \( \mu \)M) instead of iron. The samples were prepared in MEM supplemented with FBS without iron depletion.

To evaluate these parameters, the method of García-Nebot et al. (2009) was used. The cells were seeded onto polyester membrane chamber inserts (24 mm diameter, 0.4 \( \mu \)m pore size; Transwell\(^{TM}\), Costar Corp., NY, USA) at a density of 50,000 cells/cm\(^2\). The culture medium was aspirated from the apical and basolateral chambers, and washed with PBS at 37 °C. Then 2 ml
of transport buffer were added to the basolateral chamber, and 1.5 ml of samples to the apical chamber. Cell cultures were incubated at 37°C under 5% CO2 with 95% relative humidity for two hours. After incubation, the apical compartment was aspirated, the insert was removed, and the monolayer was washed with buffer transport at 4°C to remove non-specifically-bound mineral and residual medium. The cells were lysed by adding 1 ml of 2% sodium dodecyl sulphate. The basolateral chamber solution was pipetted off for the determination of zinc transport across the monolayer.

The zinc contents of the cell monolayer (zinc retention) and basolateral chamber (zinc transport) were measured by atomic absorption spectrophotometry (Perkin–Elmer, model 2380, Boston, USA). All samples were previously subjected to dry digestion at 450°C. Zinc uptake was evaluated as the sum of zinc retention and transport.

Cell protein content was determined according to Lowry, Rosebrough, Farr, and Randall (1951), for expressing the results of bioavailability, taking into account the mg of protein, and to evaluate reproducibility of the cell cultures.

### 2.4. Statistical analysis

Two-factorial ANOVA (kind of CPPs with five levels (control, pool of α-CPPs, pool of β-CPPs, αs1-CN(64–74)4P, αs2-CN(1–19)4P) and iron content (control, with iron)) was performed by mixing for 30 min at 37°C.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ferritin/mg protein</th>
<th>µg Zn/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng</td>
<td>µg</td>
</tr>
<tr>
<td></td>
<td>Retention</td>
<td>Transport</td>
</tr>
<tr>
<td><strong>Without iron/zinc</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.00 ± 1.02</td>
<td>1.32 ± 0.37</td>
</tr>
<tr>
<td>α-CPPs</td>
<td>4.73 ± 1.39</td>
<td>1.86 ± 0.35</td>
</tr>
<tr>
<td>αs1-CN(64–74)4P</td>
<td>7.84 ± 2.35</td>
<td>2.09 ± 0.24</td>
</tr>
<tr>
<td>αs2-CN(1–19)4P</td>
<td>10.9 ± 2.56</td>
<td>1.66 ± 0.25</td>
</tr>
<tr>
<td>β-CPPs</td>
<td>5.76 ± 1.73</td>
<td>1.55 ± 0.35</td>
</tr>
<tr>
<td>β-CN(1–25)4P</td>
<td>11.99 ± 5.08</td>
<td>1.75 ± 0.14</td>
</tr>
<tr>
<td><strong>With iron/zinc</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.39 ± 2.71</td>
<td>1.95 ± 0.30</td>
</tr>
<tr>
<td>α-CPPs</td>
<td>12.25 ± 3.24</td>
<td>2.66 ± 0.34</td>
</tr>
<tr>
<td>αs1-CN(64–74)4P</td>
<td>27.49 ± 6.39</td>
<td>2.21 ± 0.39</td>
</tr>
<tr>
<td>αs2-CN(1–19)4P</td>
<td>26.26 ± 7.84</td>
<td>1.53 ± 0.14</td>
</tr>
<tr>
<td>β-CPPs</td>
<td>9.53 ± 2.68</td>
<td>1.74 ± 0.37</td>
</tr>
<tr>
<td>β-CN(1–25)4P</td>
<td>36.57 ± 10.51</td>
<td>1.70 ± 0.31</td>
</tr>
</tbody>
</table>

Results are expressed as means ± standard deviation (n = 8 in two independent assays).

* a Caco-2 cells.
  b Caco-2 cells with iron or zinc sulphate.
  c Mixture of CPPs (12.5 µM) with iron or zinc (50 µM) performed by mixing for 30 min at 37°C.

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Fig. 1. Influence of CPPs on ferritin synthesis in Caco-2 cells. (a) ANOVA factors: kind of CPPs and iron content; and (b) interaction between kind of CPPs and iron content factors (p = 0.0000). Different letters (a–c) indicate statistically significant differences taking into account kinds of CPPs (p = 0.0000) or iron content (p = 0.0000) factors.
and iron or zinc contents, with two levels (with/without iron or zinc), was applied to the results obtained, followed by the Tukey post hoc test (p < 0.05). The significances of mean effects and interactions were also included (Statgraphics Plus v. 5.1, MD, USA).

3. Results and discussion

3.1. Influence of \( \alpha \)-CPPs and \( \beta \)-CPPs, and of specific CPPs, upon iron bioavailability

The results obtained, referred to ferritin synthesis in Caco-2 cells, are shown in Table 1. The treatment of Caco-2 cells with the pools of \( \alpha \)-CPPs and \( \beta \)-CPPs did not significantly increase (p > 0.05) ferritin synthesis versus control cells (see Fig. 1a). However, the three specific CPPs (\( \alpha_{s1}-CN(64-74)4P \), \( \alpha_{s2}-CN(1-19)4P \), and \( \beta-CN(1-25)4P \)) significantly increased (p < 0.05) ferritin synthesis in Caco-2 cells versus control cells, \( \beta-CN(1-25)4P \) being the most effective (see Fig. 1a). To date, no studies have evaluated the influence of CPPs from different CN fractions or specific CPPs on ferritin synthesis. In Caco-2 cells, the iron bound to \( \beta-CN(1-25)4P \) decreased iron transport and increased iron retention and uptake versus iron gluconate (Kibangou et al., 2005a), whereas it increased iron transport (Kibangou et al., 2005b, 2008) and decreased iron retention and uptake (Kibangou et al., 2008) versus iron sulphate. It has been indicated that the Fe-\( \beta-CN(1-25)4P \) complex decreases iron uptake versus iron sulphate (Kibangou et al., 2008), in disagreement with our own results. Differences between the above study and our own work can be due to the use of different CPP concentrations (25 versus 12.5 \( \mu M \)) and the measurement of iron bioavailability (iron uptake versus ferritin synthesis).

The positive influence of \( \beta-CN(1-25)4P \) on iron bioavailability, observed in this study, agrees with the findings in rats (Aït-Oukhatar et al., 1997, 1999), and by using a perfused rat duodenal loop system, Fe-\( \beta-CN(1-25)4P \) was seen to increase iron absorption versus iron gluconate (Ani-Kibangou et al., 2005; Kibangou et al., 2005a; Pérès et al., 1997, 1999a, 1999b) or ascorbate (Aït-Oukhatar et al., 2002). These authors attributed this result to resistance of the complex to enzyme hydrolysis, and the allowance of iron uptake by endocytosis together with \( \beta-CN(1-25)4P \).

In Caco-2 cells, the iron bound to a mixture of \( \alpha_{s1}-CN(59-79)5P \) and \( \alpha_{s2}-CN(2-21)4P \) (Kibangou et al., 2005a) or to \( \alpha_{s1}-CN(59-79)5P \) (Kibangou et al., 2005b) decreased iron retention, transport and uptake versus Fe-\( \beta-CN(1-25)4P \). This agrees with our own observations, in which the pool of \( \alpha-CPPs \) or \( \alpha_{s1}-CN(64-74)4P \) or \( \alpha_{s2}-CN(1-19)4P \) decreased ferritin synthesis versus \( \beta-CN(1-25)4P \) (see Fig. 1a). Despite the fact that the CPP sequence...
employed was similar (\(\alpha_{12}\)-CN\((59–79)5P\) versus \(\alpha_{12}\)-CN\((64–74)4P\)), when the effect of the complex is compared with iron sulphate, the above-mentioned study (Kibangou et al., 2005b) showed a decrease in iron transport and uptake. However, we observed an increase of ferritin synthesis in Caco-2. The differences in results between the two studies could be attributable to the different CPP concentrations used (25 versus 12.5 \(\mu\)M) and the measurement of iron bioavailability (iron uptake versus ferritin synthesis).

In a perfused duodenal loop system, Bouchallab et al. (2002) reported greater uptake and net absorption of iron from fraction \(\beta\)-CN, containing \(\beta\)-CN\((1–25)4P\), versus \(\alpha\)-CN, thus suggesting that the efficiency of various CPPs on iron absorption depends on their origin and structural properties. Different conformations were reported for three specific CPPs (\(\beta\)-CN\((1–25)4P\), \(\alpha_{11}\)-CN\((59–79)5P\) and \(\alpha_{12}\)-CN\((2–20)4P\)), in the presence of calcium, which present the common cluster (SpSpSpEE) (Huq, Cross, & Reynolds, 2003) – a fact attributed to the influence which the residues neighbouring the cluster could exert upon conformation. This fact could explain the differences in ferritin synthesis found in Caco-2 cells among \(\beta\)-CN\((1–25)4P\), \(\alpha_{11}\)-CN\((64–74)4P\) and \(\alpha_{12}\)-CN\((1–19)4P\) (Huq, Cross, & Reynolds, 2003).

The addition of iron significantly increased \((p < 0.05)\) ferritin synthesis in Caco-2 cells (see Fig. 1a), in accordance with the observations of Glahn, Wortley, South, and Miller (2002), who reported an increase in ferritin synthesis from iron chloride between 0 and 50 \(\mu\)M. The increase in ferritin synthesis due to the complexes between the pools of \(\alpha\)-CPPs or \(\beta\)-CPPs with iron did not differ from that associated with iron sulphate in Caco-2 cells (control cells). In contrast, \(Fe-\beta\)-CN\((1–25)4P\), \(Fe-\alpha_{11}\)-CN\((64–74)4P\) and \(Fe-\alpha_{12}\)-CN\((1–19)4P\) increased ferritin synthesis (see Fig. 1b).

### 3.2. Influence of \(\alpha\)-CPPs, \(\beta\)-CPPs, and specific CPPs, on zinc bioavailability

The results obtained in relation to zinc retention, transport and uptake in Caco-2 cells are shown in Table 1. The \(\alpha\)-CPPs pool significantly increased \((p < 0.05)\) zinc retention, \(\beta\)-CPPs significantly increased \((p < 0.05)\) zinc transport, and both pools significantly increased \((p < 0.05)\) zinc uptake versus control cell (see Fig. 2a).

The positive influence of the pool of \(\alpha\)-CPPs versus the pool of \(\beta\)-CPPs on zinc retention could be attributed to greater binding of CPPs proceeding from the \(\alpha\)-CN versus the \(\beta\)-CN fractions. In contrast, in a previous study by our group, it was seen that zinc showed greater binding to CPPs released from infant formulas subjected to simulated gastrointestinal digestion, and proceeding from \(\alpha\)-CN versus \(\beta\)-CN fractions (Miquel et al., 2005).

The positive influence of the pool of \(\beta\)-CPPs on zinc transport could be attributed to the absorption process mediated by endocytosis, as indicated for the \(Zn-\beta\)-CN\((1–25)4P\) complex in a perfused duodenal loop system (Pérès et al., 1998). Likewise, in Caco-2 cells, it has been shown that one peptide of 17 residues is transported by transcytosis, which includes an endocytic process (Regazzo et al., 2010). Thus, zinc could be transported bound to CPPs by means of this mechanism.

The \(\alpha_{11}\)-CN\((64–74)4P\) significantly increased \((p < 0.05)\) zinc retention versus control cells. For zinc transport and uptake, there were statistically significant differences \((p < 0.05)\) in the following order: \(\alpha_{11}\)-CN\((64–74)4P > \beta\)-CN\((1–25)4P > \alpha_{12}\)-CN\((1–19)4P\) (see Fig. 2a).

The positive influence of \(\beta\)-CN\((1–25)4P\) on zinc uptake agrees with the observations of Pérès et al. (1998), who attributed this result to a protective effect of zinc bound to \(\beta\)-CN\((1–25)4P\) against insolubilisation during digestion or interactions with other components in the gut.

The differences in conformation acquired by the complex when the CPPs bind to zinc could explain the differences found in zinc retention, transport and uptake in Caco-2 cells among \(\beta\)-CN\((1–25)4P\), \(\alpha_{11}\)-CN\((64–74)4P\) and \(\alpha_{12}\)-CN\((1–19)4P\).

It must be pointed out that this is the first time an evaluation has been made of the influence of specific CPPs upon zinc retention, transport and uptake in Caco-2 cells, \(\alpha_{11}\)-CN\((64–74)4P\) being the most effective.

The addition of zinc significantly increased \((p < 0.05)\) zinc retention, transport and uptake (see Fig. 2b). This result agrees with the increase in zinc retention and transport in Caco-2 cells from zinc solutions at concentrations from 0 to 200 \(\mu\)M (Finley, Briske-Anderson, Reeves, & Johnson, 1995; Iyengar, Pullakhandam, & Nair, 2009; Sreenivasulu, Raghu, Ravinder, & Nair, 2008).

When the interactions between the kind of CPPs and zinc content were evaluated, we observed that the effect of zinc addition varies, depending on the sample. Complexes \(Zn-\alpha\)-CPPs, \(Zn-\beta\)-CPPs and \(Zn-\alpha_{11}\)-CN\((64–74)4P\) increased zinc uptake. In the case of \(Zn-\alpha\)-CPPs, this effect was due to an increase in zinc retention. In contrast, in the case of \(Zn-\beta\)-CPPs and \(Zn-\alpha_{11}\)-CN\((64–74)4P\), the effect was due to increased zinc transport. Despite these results, it must be pointed out that no sample exerted a greater influence on zinc uptake than did zinc sulphate (see Fig. 3a-c).

### 4. Conclusion

The treatment of Caco-2 cells with the pools of \(\alpha\)-CPPs and \(\beta\)-CPPs did not improve iron bioavailability (ferritin synthesis). In contrast, the three specific CPPs (\(\alpha_{11}\)-CN\((64–74)4P\), \(\alpha_{12}\)-CN\((1–19)4P\) and \(\beta\)-CN\((1–25)4P\)) increased ferritin synthesis, \(\beta\)-CN\((1–25)4P\) being the most effective. These results suggest that the efficiency of various CPPs on ferritin synthesis depends on their
structural properties and on the conformation which the complex can acquire when iron is bound to CPP.

$\alpha_3$-CN(64–74)4P increased zinc retention, transport and uptake, being the most effective CPP in this context. However, from the results obtained, it can be concluded that the effect on zinc retention, transport and uptake attributable to the addition of zinc in the form of complexes with the pools of CPPs or specific CPPs does not represent an improvement with respect to zinc addition in the form of sulphate.

Further in vitro and in vivo studies are thus needed to elucidate the ways in which specific CPPs improve iron and zinc bioavailability in order to use them as functional components.

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