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## REDUCTION OF IRON/ZINC INTERACTIONS USING METAL BOUND TO THE CASEINOPHOSPHOPEPTIDE 1-25 OF $\beta$ -CASEIN

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### ABSTRACT

We used the isolated, perfused rat duodenal loop system to assess the influence of binding iron (Fe) to soluble 1-25 caseinophosphopeptide ( $\beta$ -CN (1-25)), produced by the hydrolysis of  $\beta$  casein, against the inhibition of its absorption by Zinc (Zn). Fe (100  $\mu$ M) was perfused as Fe gluconate (Fe Gluc) or bound to the  $\beta$ -CN (1-25) (Fe-CN), alone (controls) or in presence of Zn as Zn sulfate (Zn SO<sub>4</sub>) or as (Zn-CN) at Fe:Zn ratios ranging from 2:1 to 1:5. Zn SO<sub>4</sub> reduced significantly disappearance from the lumen (Q1) and net Fe absorption (FeAbs) at Fe:Zn ratios from 1:1.5 to 1:5 ( $p < 0.001$ ). Fe mucosal retention (Q2) did not change significantly. When Zn was provided as Zn-CN, Q1, Q2 and Fe Abs did not significantly differ from control group for Fe Gluc. Zn SO<sub>4</sub> and Zn-CN did not reduce significantly Q1, Q2 nor FeAbs for Fe-CN whatever ratios considered. Binding Fe to  $\beta$ -CN (1-25) prevented Zn from inhibiting its absorption by Zn and could have therapeutic applications in dietary supplementation of trace-elements.

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**KEY WORDS :** Iron, Zinc, Iron-Zinc Interactions, Digestive Absorption Caseinophosphopeptide, Rat.

### INTRODUCTION

Iron deficiency is the first cause of nutritional anemias. In fact, iron bioavailability is usually low and largely depends on several factors like intestinal pH, reducing agents and inhibitory compounds: Ca inhibits Fe absorption (1,2) and Fe impairs Zn absorption and metabolism (3-7). An inhibitory influence of Zn supply on Fe absorption has been less frequently reported however (2,8-10). So far the mechanisms involved in these interactions are not precisely defined: Zn and Fe have similar physicochemical properties (10) and compete for a common transport mechanism at the enterocyte membrane (11-13); inhibitory interactions could also occur during trace element transfer from apical membrane to membrane (14).

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Previous studies showed that binding Fe to  $\beta$ -CN (1-25) enhances its absorption and prevents the interactions between Fe and Ca or Fe and Zn (5,15).  $\beta$ -CN (1-25) is a well defined phosphopeptide produced by the hydrolysis of  $\beta$ -casein, which has four of the five phosphoserine residues of the native protein. These residues can bind divalent cations such as Fe, Zn or calcium in proportion to their degree of phosphorylation and according to the cation (16-18). Thus 1 mole of  $\beta$ -CN (1-25) can bind 4 moles of Fe or Zn with a greater affinity than for calcium (18).

The present study used the isolated duodenal rat loop model to assess Zn upon Fe interactions (at ratios usually observed during drug supplementation and food fortification), and the influence of binding trace elements to  $\beta$ -CN (1-25) on these interactions. Fe was supplied in perfusion as Fe Gluc or bound to  $\beta$ -CN (1-25); Zn was bound to  $\beta$ -CN (1-25) (Zn-CN) or Zn sulphate (Zn SO<sub>4</sub>).

### METHODS AND MATERIALS

#### Preparation of the 1-25 caseinophosphopeptide of $\beta$ -casein ( $\beta$ CN (1-25))

$\beta$  Casein was isolated from industrially-produced sodium caseinate (Armor Protéines, Saint-Brice-en-Coglès, France) by cold solubilization (pH 4,5 ; 4°C), followed by ion exchange chromatography (15,17,19).  $\beta$ -CN (1-25) was obtained by tryptic digestion of  $\beta$  casein. Fe and Zn were bound to  $\beta$ -CN (1-25) by adding FeCl<sub>2</sub> or ZnCl<sub>2</sub> to the solution (pH=5.3 ; 30 min ; 25°C (Milli Q system, Millipore)). Unbound Fe and Zn were removed by ultracentrifugation and filtration through a regenerated cellulose membrane with a 3000 Da cut off (membrane SIOY3, Amicon, Lexington, MY). The resulting complex was freeze-dried. Complexed Fe and Zn and residual calcium were measured by atomic absorption spectrometry (Varian AA 1275). One mole of peptide bound 4 moles of Fe or Zn. A control without  $\beta$ -CN (1-25) was incubated and dialysed under the same conditional, to be sure that the variations of Zn and Fe concentrations were actually induced by binding to the phosphopeptide.

#### Animals

Adult female Sprague-Dawley rats weighing 250 to 300g were fed prior to the study with a semi synthetic diet containing iron and zinc in normal quantities. They were starved for 12 hours before the study but had free access to water.

#### Perfusion

The perfusion solute was adapted from Ringer-Lavoisier solute and was checked to be free of Fe and Zn contamination. Its pH was adjusted to that of proximal duodenum pH (pH=4-4,5); it was isotonic to plasma (285-300 mosmol) and contained 100  $\mu$ mol/L Fe as gluconate or Fe-CN. Rats were anaesthetised with Ketamine (Ketalar<sup>TM</sup>). Then the duodenum was exposed by a laparotomy. The loop was perfused through a catheter inserted into the pylorus ; effluent was collected at the angle of Treitz. Solid material was washed out with 1 g/l Triton X100 in water to prevent any contamination. The perfusion solute was kept at 37°C and was delivered at 0.16 ml/min for 2 hours using a peristaltic pump to avoid loop distension. A non-absorbable marker (polyethylene glycol 4000) was added to the solute to assess net water fluxes. At the end of the experiment, rats were killed by an overdose of Doléthal<sup>TM</sup>. The perfused loop was washed with saline, withdrawn and dried in an oven at 90°C to constant weight.

Rats were assigned to 18 groups (6 animals/group) and perfused with 100  $\mu\text{mol/L}$  Fe gluconate (Fe Gluc) (which remains soluble above  $\text{pH}=4$ ), or Fe bound to  $\beta\text{-CN}$  (1-25) (Fe-CN); Fe was perfused alone (controls) or in presence of Zn as Zn sulphate ( $\text{Zn SO}_4$ ), or Zn bound to  $\beta\text{-CN}$  (1-25) (Zn-CN). Zn concentrations were 0  $\mu\text{mol/L}$ , 50  $\mu\text{mol/L}$ , 100  $\mu\text{mol/L}$  and 500  $\mu\text{mol/L}$  when Fe or Zn were bound to  $\beta\text{-CN}$  (1-25) giving Fe:Zn ratios of 2:1, 1:1 and 1:5. When Fe and Zn were as free salts, Zn concentrations were 0  $\mu\text{mol/L}$ , 50  $\mu\text{mol/L}$ , 100  $\mu\text{mol/L}$ , 150  $\mu\text{mol/L}$ , 200  $\mu\text{mol/L}$ , 300  $\mu\text{mol/L}$ , 400  $\mu\text{mol/L}$  and 500  $\mu\text{mol/L}$  giving Fe:Zn ratios of 2:1, 1:1, 1:1.5, 1:2, 1:3, 1:4 and 1:5.

The absence of Fe adsorption to the digestive mucosa, which could falsely improve the absorption rate, was checked by perfusing a group of dead rats. Another group of rats was perfused with a Fe-free solution to confirm that no significant Fe secretion occurred during the experiment.

### Assays

Fe and Zn concentrations in perfusion solute, gut effluent and the mucosa of the perfused segment were measured by atomic absorption (Perkin Elmer 3030). The tissue was digested in 10 N nitric acid at ambient temperature for 24 hours. Ringer-Lavoisier solute was used as blank. Polyethyleneglycol (PEG) in the perfusion solute and the gut effluent was measured by a turbidimetric method (20).

The disappearance of Fe from the gut lumen ( $Q_1$ :  $\mu\text{mol}$ ) was calculated as follows:

$$Q_1 = (1 - ([\text{PEG}]_t / [\text{PEG}]_e)) * ([\text{Fe}]_e / [\text{Fe}]_t) * D * T * [\text{Fe}]_t$$

where  $[\text{PEG}]$  and  $[\text{Fe}]$  were the PEG and Fe concentrations in the perfusion solute (t) and in the effluent (e). D and T are the delivery rate (ml/min) and the time of collection.

The Fe stored by the mucosa ( $Q_2$ :  $\mu\text{mol}$ ) during perfusion was calculated from:

$$Q_2 = ([\text{Fe}]_m - [\text{Fe}]_{m0}) * P_m$$

where  $[\text{Fe}]_m$  is the Fe concentration of perfused intestinal mucosa segment and  $P_m$  its weight ;  $[\text{Fe}]_{m0}$  the Fe concentration of intestinal mucosa segment perfused with a Fe-free solute.

Net Fe absorption (Fe abs:  $\mu\text{mol}$ ) during the perfusion was :

$$\text{Fe abs} = Q_1 - Q_2.$$

### Statistics

Results are expressed as means and standard deviations. Groups were compared by two ways-ANOVA followed by Student's t-test using "Statview SE + Graphics™" within each Fe group (Fe Gluc or Fe-CN). Results of Zn perfused animals were expressed as a ratio of the mean for the control Zn-free group (%). These values were also compared by ANOVA and Student's t-test. Absorption of Fe gluc or Fe-CN was improved by Student's t-test. Significance was set at  $p < 0.05$ .

## RESULTS

The influence of different forms of Zn on Fe Gluc and Fe-CN absorption are shown in Tables 1 and 2 respectively.

Fe-CN absorption was significantly higher than Fe Gluc absorption when Fe was perfused alone ( $p < 0.001$ ). This absorption remained significantly higher whatever zinc concentration considered ( $p < 0.001$ ).

At low concentrations (Fe:Zn ratio : 2:1 ; 1:1), Zn SO<sub>4</sub> did not reduce Q1, Q2 and FeAbs of Fe Gluc ( table 1). Higher concentrations of Zn SO<sub>4</sub> significantly reduced significantly Q1 and FeAbs ( $p < 0.001$ ). This inhibition increased significantly for higher Zn SO<sub>4</sub> concentrations ( $p < 0.001$ ). On the contrary, Zn-CN had no effect on Q1, Q2 and FeAbs whatever Fe:Zn ratio considered.

TABLE 1

Effect of Zinc on Iron Gluconate Absorption

	Fe:Zn Ratio	%Q1	%Q2	% FeAbs	Inhibition of FeAbs
Fe Gluc (100µM)		45.9 ± 0.4	8.5 ± 1.9	37.4 ± 1.9	
+ Zn SO <sub>4</sub> (50µM)	2:1	47.7 ± 1.3	8.4 ± 1.3	39.3 ± 2.5	-5.1%
+ Zn SO <sub>4</sub> (100µM)	1:1	46.5 ± 1.2	8.8 ± 2.3	37.7 ± 2.0	-1.1%
+ Zn SO <sub>4</sub> (150µM)	1:1.5	35.1 ± 2.0 <sup>*#</sup>	9.0 ± 1.8	26.1 ± 3.1 <sup>*#</sup>	30.3%
+ Zn SO <sub>4</sub> (200µM)	1:2	30.2 ± 0.5 <sup>*#</sup>	9.2 ± 1.0	21.0 ± 1.1 <sup>*#</sup>	43.9%
+ Zn SO <sub>4</sub> (300µM)	1:3	28.1 ± 0.6 <sup>*#</sup>	9.0 ± 1.3	19.1 ± 1.7 <sup>*#</sup>	49.0%
+ Zn SO <sub>4</sub> (400µM)	1:4	27.6 ± 1.4 <sup>*</sup>	8.6 ± 2.0	19.0 ± 2.4 <sup>*</sup>	49.2%
+ Zn SO <sub>4</sub> (500µM)	1:5	27.1 ± 1.4 <sup>*</sup>	8.5 ± 2.7	18.6 ± 3.5 <sup>*</sup>	50.2%
ANOVA		p < 0.001	NS	p < 0.001	
+ Zn-CN (50µM)	2:1	46.3 ± 0.6	8.4 ± 3.4	37.9 ± 3.1	-1.3%
+ Zn-CN (100µM)	1:1	46.2 ± 1.7	8.4 ± 2.7	37.8 ± 3.3	-1.1%
+ Zn-CN (500µM)	1:5	46.7 ± 1.2 <sup>**</sup>	8.3 ± 2.9	38.4 ± 2.7 <sup>**</sup>	-2.7%
ANOVA		NS	NS	NS	

Results are expressed as a ratio to the total amount of perfused Fe (%); mean ± 1SD ; Rats were perfused with perfusion solute containing Fe Gluconate (Fe Gluc) (100 µmol/L) ; Zn sulfate (Zn SO<sub>4</sub>) or Zn bound to β-CN (1-25) (Zn-CN) were added at different concentrations. Q1 : Fe lost from gut lumen ; Q2 : Fe stored by the mucosa ; FeAbs : net Fe absorption . Statistical analysis: 1) between groups perfused with the same form of Zn; ANOVA followed by Student's t tests; \* : different ( $p < 0.001$ ) from control group Fe Gluc ; # : different ( $p < 0.001$ ) from previous group; 2) Student's t tests between groups perfused with different forms of Zn at same concentration; \*\* : different ( $p < 0.001$ ) from ZnSO<sub>4</sub>.

Inhibition: % inhibition of the mean value of the control group, perfused without Zn.

The influence of the form of Zn on Fe absorption was assessed by comparing groups perfused with the two forms of Fe at same Zn concentrations: Q1 and FeAbs were significantly different between Zn SO<sub>4</sub> and as Zn-CN for a Fe:Zn ratio of 1:5.

Table 2 shows that neither Zn SO<sub>4</sub> nor Zn-CN had any significant effect on Q1, Q2 and Fe Abs at any Fe:Zn ratio.

TABLE 2  
Effect of Zinc on Absorption of Iron Bound to  $\beta$ -CN (1-25)

	Fe:Zn Ratio	%Q1	%Q2	% FeAbs	Inhibition of FeAbs
Fe-CN (100 $\mu$ M)		55.0 $\pm$ 0.5	7.6 $\pm$ 1.6	47.4 $\pm$ 1.8	
+ Zn SO <sub>4</sub> (50 $\mu$ M)	2:1	54.9 $\pm$ 0.9	7.8 $\pm$ 1.5	47.1 $\pm$ 1.5	0.6%
+ Zn SO <sub>4</sub> (100 $\mu$ M)	1:1	54.3 $\pm$ 1.4	7.3 $\pm$ 1.8	47.0 $\pm$ 1.4	0.8%
+ Zn SO <sub>4</sub> (500 $\mu$ M)	1:5	52.7 $\pm$ 1.9	7.6 $\pm$ 1.9	45.1 $\pm$ 1.8	4.9%
ANOVA		NS	NS	NS	
+ Zn-CN (50 $\mu$ M)	2:1	56.2 $\pm$ 1.4	8.0 $\pm$ 1.4	48.2 $\pm$ 0.2	-1.7%
+ Zn-CN (100 $\mu$ M)	1:1	56.4 $\pm$ 1.8	8.1 $\pm$ 2.8	48.3 $\pm$ 3.1	-1.9%
+ Zn-CN (500 $\mu$ M)	1:5	55.8 $\pm$ 2.0	8.1 $\pm$ 2.0	47.7 $\pm$ 3.7	-0.6%
ANOVA		NS	NS	NS	

Results are expressed as a ratio to the total amount of perfused Fe (%); mean  $\pm$  1SD ; Rats were perfused with perfusion solute containing Fe bound to  $\beta$ -CN(1-25) (Fe Gluc) (100  $\mu$ mol/L) ; Zn Sulfate (Zn SO<sub>4</sub>) or Zn bound to  $\beta$ -CN (1-25) (Zn-CN) was added at different concentrations. Q1 : Fe lost from gut lumen ; Q2 : Fe stored by the mucosa ; FeAbs : net Fe absorption. Statistical analysis: 1) ANOVA between groups perfused with the same form of Zn; 2) Student's t tests between groups perfused with different forms of Zn at same concentration: no statistical difference.

Inhibition: % inhibition of the mean value of the control group, perfused without Zn (%).

Figure 1 shows the results expressed as the % inhibition of FeGluc absorption by Zn SO<sub>4</sub>. This inhibition was significantly greater using Fe Gluc at Fe:Zn ratios ranging from 1:1.5 to 1:3, then plateaued from the ratio 1:3 to 1:5. The others groups were not shown because of the lack of significant inhibition : no interactions were observed when Fe or Zn was provided in a bound form.

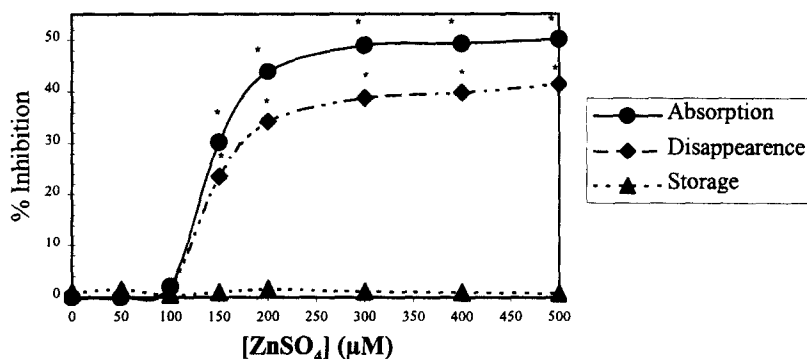


FIG. 1. Inhibition of intestinal absorption of Fe Gluc (100  $\mu$ M) by Zn SO<sub>4</sub> (50-500  $\mu$ M). Results are expressed as % inhibition of the mean value of the control group, perfused without Zn (%); \*  $p < 0.001$  versus control.

## DISCUSSION

We come to a better knowledge of incidence and functional consequences of Zn deficiency. Yet Fe:Zn inhibition is better known than Zn:Fe interactions, the latter ones are suggested by some experimental, human and in vitro studies (4). Inhibition of Fe absorption by Zn could create or exacerbate Fe deficiency in as much as several associated deficiencies are usually found in the same subject (9,21).

This study was designed to determine at which Fe:Zn ratio interactions could occur and how Fe absorption could be protected against those interferences. Our results showed that the inhibition of Fe Gluc absorption by Zn SO<sub>4</sub> appears at ratio 1:1.5. On the other hand when either trace element was perfused bound to  $\beta$ -CN (1-25) no such inhibition was observed even at Fe:Zn ratio 1:5. These results are in agreement with previous studies which failed to display interactions between Fe and Zn when provided by diet at usual dietary levels (9,22). The diet should provide similar amounts (12mg in the adult) of Fe and Zn, as stated by the RDA (23). On the other hand, our results support previous reports which displayed interactions between Zn and Fe when provided at higher ratios and in a solute form (9,22,24). Therefore higher than dietary levels of Zn are likely to exacerbate Fe deficiency. The protection offered by binding trace element to  $\beta$ -CN (1-25) could prevent these interactions. Yet the susceptibility of Zn-CN to hydrolysis is not currently defined, it is known that Fe-CN binding resists enzyme digestion. Phosphoserine residues of phosphoproteins such as caseins or phosphopeptides issued from casein enzyme hydrolysis are strong ligands for divalent cations (16,17,25) and keep them soluble in the digestive lumen. Caseinophosphopeptides are stable compounds which are usually found in the intestinal lumen after milk or yoghurt ingestion in man (26,27) and do not present technological hazards as they are dietary food. It has been previously shown that binding Fe to  $\beta$ -CN (1-25) of  $\beta$  casein enhances its digestive absorption (15) and its ability to cure Fe deficiencies (28) as well as Zn absorption (5). Furthermore, it prevents the inhibitive interactions of Ca on Fe and of Fe on Zn (5,15). The present study showed that binding Zn or Fe to  $\beta$ -CN (1-25), significantly reduced their interactions whatever Fe:Zn ratio considered, even at extreme values (Fe:Zn=1:5). The improved bioavailability of  $\beta$ -CN (1-25) bound Zn and Fe could be explained by the presence of trace-element peptides complexes in the lumen which are resistant to enzyme hydrolysis: so that binding trace elements to caseinophosphopeptides could improve their bioavailability as compared to just adding them to a blend of peptides (29,30). The bound form of trace elements could also enhance their digestive absorption by preventing interactions during membrane transfer since caseinophosphopeptide bound Fe and Zn are partly absorbed by specific pathways, including endocytosis, which do not share the same transport mechanisms as free ions (5,31).

## CONCLUSION

This study confirms the enhanced absorption of trace elements when provided bound to  $\beta$ -CN (1-25). Furthermore binding trace elements to  $\beta$ -CN (1-25) protects it from inhibitory interactions with other divalent cations. Further studies are needed to validate those conclusions in man and to better define mechanisms involved in this protection.

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