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Iron Tissue Storage and Hemoglobin Levels of Deficient Rats Repleted with Iron Bound to the Caseinophosphopeptide 1–25 of β -Casein

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Caseinophosphopeptides (CPP) issued from enzyme digestion of caseins bind cations and keep them soluble in the digestive tract. They could be used as ligands to improve iron (Fe) bioavailability. Fe-deficient young rats were repleted with Fe (40 or 200 mg/kg of diet) bound either to the β -CN (1–25) of β -casein or to whole β -casein or as FeSO₄. A control pair-fed group was given 200 mg of Fe (FeSO₄)/kg of diet for 6 weeks. After repletion, hemoglobin concentration of the control group was reached only by the β -CN (1–25) animals fed 200 mg of Fe/kg; β -CN (1–25) bound Fe (40 and 200 mg) produced higher Fe liver and spleen stores than FeSO₄. Binding Fe to the whole, nonhydrolyzed β -casein gave results intermediate between the other experimental groups. Binding Fe to phosphoserine residues of low molecular weight CPP improved its ability to cure anemia and to restore iron tissue stores, as compared to Fe bound to the whole casein and to inorganic salts.

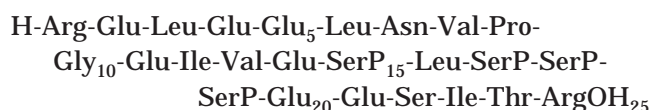
Keywords: Iron; bioavailability; tissue storage; hemoglobin; casein phosphopeptide; milk proteins; rat

INTRODUCTION

Iron (Fe) absorption is usually poor and is influenced by interactions with other food components: Proteins can enhance or decrease Fe absorption depending on their origin; soy and other vegetal proteins usually reduce Fe absorption, even if phytates are removed from vegetal proteins, although there are some conflicting reports (Cook and Monsen, 1976; Hurrell et al., 1989, 1992; Thompson and Erdman, 1984; Wapnir, 1990). Animal foods such as beef, pork, chicken, and fish are enhancing factors due to their content in cysteine (Cook and Monsen, 1976; Martinez-Torres et al., 1981). Heme-bound iron is soluble at the alkaline pH of proximal bowel and is absorbed by a specific pathway, which results in a high absorption rate (Beard, 1996). Eggs, milk, and milk products such as cheese are assumed to be inhibitors of non-heme Fe absorption (Beard, 1996; Cook and Monsen, 1976; Hurrell et al., 1988, 1989; Wapnir, 1990); their phosphoproteins strongly bind transition metals by ionic interactions with their phosphoserine residues (Brulé et al., 1979; Demott and Dincer, 1976; Hegenauer et al., 1979; West, 1986); therefore, they could release Fe too far down the intestine for efficient absorption (Miller and Berner, 1989; West, 1986). Binding Fe to low molecular weight,

soluble peptides could overcome this pitfall and favor its absorption.

Caseinophosphopeptides (CPP) are yielded by *in vitro* (Juillerat et al., 1989) or *in vivo* (Kasai et al., 1995; Meisel and Frister, 1989; Naito et al., 1972) enzyme cleavage of casein and are considered to be stable to further proteolysis by digestive enzymes (Kasai et al., 1995). The 25 amino acid CPP issued from tryptic hydrolysis of β -casein [β -CN (1–25)] contains four of the five phosphoserine residues of the native protein (Juillerat et al., 1989; Manson and Annan, 1971; West, 1986):



CPP have a high capacity to bind divalent cations such as calcium, Fe, zinc, and copper and keep them soluble at luminal pH (Berrocal et al., 1989; Galdi and Valencia, 1988; Meisel and Frister, 1989; Sato et al., 1991); affinity of caseins is 50–100-fold higher for Fe than for calcium or magnesium (Brulé and Fauquant, 1982).

Binding Fe to β -CN (1–25) enhances its digestive absorption and prevents the inhibitory effect of calcium (Pèrés et al., 1997, 1998).

In the present study it was assumed that binding Fe to this low molecular weight phosphopeptide could improve also its ability to restore hemoglobin levels and tissue storage of the Fe-deficient animal as compared to a free Fe salt (FeSO₄) or to Fe bound to the large intact β -casein. Two doses of Fe were used: the lower one (40 mg/kg of diet) is based on the nutrient requirements for rats (National Research Council, 1995); the higher one (200 mg/kg of diet) intends to mimic the amount of Fe supplied by fortified products.

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Table 1. Composition of the Diet^a

nutrient		nutrient	
protein (%)	20	minerals and vitamins (%)	5.5
lipids (%)	4.4	iron (mg/kg)	5–40, 200
carbohydrates (%)	56.4	moisture (%)	9.7
cellulose (%)	4.3		

^a Diet deprived of protein (UAR Aprotéique) to which casein or casein + β -casein derived peptides were added to a final level of 20%. Components of UAR Aprotéique: proteins, 0%; glucose + starch, 80%; cellulose, 6%; lipids, 6%; (minerals) P, 7.75 g/kg; Ca, 10 g/kg; K, 6 g/kg; Na, 4 g/kg; Mg, 1 g/kg; Mn, 80 mg/kg; Zn, 45 mg/kg; Cu, 12.5 mg/kg; (vitamins) A, 19800 UI/kg; D, 2500 UI/kg; B1, 20 mg/kg; B2, 15 mg/kg; B6, 10 mg/kg; E, 170 mg/kg; K, 40 mg/kg; PP, 100 mg/kg; biotin, 0.3 mg/kg; folic acid, 5 mg/kg.

MATERIALS AND METHODS

Protein and Peptide Source. β -Casein was isolated from industrially made sodium caseinate (Armor Protéines, St-Brice-en Coglès, France) using its solubility at pH 4.5 at 4 °C followed by ion exchange chromatography (Baumy and Brulé, 1988; Bouhallab et al., 1991). The β -CN (1–25) was prepared using a tryptic hydrolysis of β -casein (Brulé et al., 1979).

β -CN (1–25) was then incubated at a concentration of 12.5 mg/mL (7×10^{-6} mol/L) with 10-fold excess of FeCl₂ solution (4×10^{-2} mol/L, pH 5.3) during 30 min at 25 °C (Milli-Q system, Millipore). The preparation was dialyzed during 24 h at 4 °C to remove unbound Fe, using dialysis bags with a cutoff of 1000 Da. Binding Fe to the β -casein was performed in the same way, but concentrations of β -casein and of FeCl₂ were 5.2×10^{-4} mol/L (12.5 mg/mL) and 2.6×10^{-3} mol/L, respectively. The amount of Fe complexed to β -CN (1–25) or to β -casein was determined by atomic absorption spectrometry (Varian; model AA 1275). A dialysis control without β -CN (1–25) or protein was performed.

Four moles of Fe were bound per mole of β -CN (1–25) and 5 mol of Fe per mole of the native β casein. Fe is bound to phosphoserine residues in a ferric form (Emery, 1992).

Experimental Design. Weaning male Sprague Dawley rats (issued from the farm of the University of Caen), 23 days old, weighing 50–60 g, were housed individually in plastic and stainless steel wire bottom metabolic cages. They were divided into six groups of eight rats. Five groups were fed an Fe-deficient diet (<5 mg of Fe/kg) containing 20% protein as casein (UAR, Villemaison-sur-Orge, France) for 4 weeks. Rats had free access to diet and distilled water. At the end of this depletion period, these five deficient experimental groups were fed the same Fe-deficient diet to which Fe had been added at respective concentrations of 40 and 200 mg/kg. Fe sources were Fe²⁺SO₄ [groups (FeSO₄ 40 mg) and (FeSO₄ 200 mg)], Fe bound to β -CN (1–25) [groups β -CN (1–25) 40 mg and β -CN (1–25) 200 mg], and Fe bound to β -casein (group Cas-Fe 200 mg). During the 2 weeks of the repletion period the five groups had free access to diet and distilled water.

A control group (pair-fed) was pair-fed to the mean intakes of Fe-deficient groups with the standard diet (20% protein as casein; 200 mg of Fe/kg of diet as FeSO₄·7H₂O) for 6 weeks. The detailed composition of the diet is given in Table 1: casein or casein + β -casein derived peptides were added to a final level of 20% to a diet deprived of protein (UAR Aprotéique). Animals were weighed upon arrival in the laboratory and weekly thereafter.

After 4 weeks, blood was drawn for a blood cell count on a Coulter Counter S890. Plasma Fe was measured, but data were discarded due to an unpredictable degree of hemolysis that occurred during drawing blood.

At the end of the experiment, animals (65 days old) were killed by an overdose of pentobarbital; blood was drawn for another blood cell count, and liver and spleen were excised, weighed, and frozen; before analysis, the organs were dried at 90 °C, ground, and digested by nitric acid (ultrapure nitric acid, Merck) in a microwave oven (Microdigest A 301, Prolabo, France).

Fe concentration was measured by atomic absorption spectrometry (Perkin-Elmer 3030).

Bovine liver standard (National Bureau of Standards, Washington, DC, Standard Reference Material NIST 1577B) was analyzed to evaluate the methodological accuracy of the laboratory method. Certified concentration was (mean \pm 1 SD) 184 \pm 15 μ g/g; laboratory value was (n = 13) 190 \pm 6 μ g/g. The run to run coefficient of variation was 1.29% (n = 30).

Statistical Analysis. Data are expressed as means and standard errors of the mean (SEM). The experimental data were analyzed by two-way ANOVA followed by Fisher's exact tests on statView SE + Graphics, Abacus Concept, Inc.

ANOVA with repeated measures was used to assess the changes in hemoglobin levels between the beginning and the end of repletion.

Comparisons were made within the 4 groups fed 200 mg of Fe/kg and within the two groups fed 40 mg/kg; in addition, a dose effect was looked for by comparison between the two FeSO₄ groups and between the two β -CN (1–25) groups.

A probability level of p < 0.05 was considered significant.

RESULTS

During the two last weeks of the study, total weight gains were 61 \pm 6 and 81 \pm 7 g in FeSO₄ and β -CN (1–25) 40 mg Fe fed groups, respectively (t = 2.0, p = 0.06); for 200 mg groups weight gains were 92 \pm 5, 93 \pm 4, 106 \pm 4, and 110 \pm 3 g in FeSO₄, β -CN (1–25), pair-fed, and Cas-Fe groups, respectively (ANOVA, F = 5.3; Fisher's exact t test, p = 0.005); a significant difference (p < 0.05) was displayed between FeSO₄ or β -CN (1–25) and pair-fed or Cas-Fe groups; FeSO₄ did not differ from β -CN (1–25) animals nor pair-fed from Cas-Fe.

Hemoglobin (Hb) levels and red blood cell counts (RBC) at the beginning and the end of repletion period are given in Table 2. Reticulocyte counts were not performed at the end of the repletion periods because they add no information to a normal RBC count. Because of a significant difference between Hb levels of experimental groups at the end of depletion, an ANOVA with repeated measures was performed to study the changes occurring during the two repletion weeks.

The weights (g; $x \pm$ SEM) of liver were 11.3 \pm 1.0 and 15.9 \pm 1.4, respectively, in FeSO₄ and β -CN (1–25) (40 mg) animals (Fisher's exact t test, p < 0.05), and spleen weights were 0.9 \pm 0.1 and 1.1 \pm 0.1 (NS); liver weights of FeSO₄ (200 mg) animals (14.1 \pm 0.7), β -CN (1–25) (200 mg) (13.4 \pm 1.2), pair-fed (12.6 \pm 0.8), and Cas-Fe (14.5 \pm 0.8) did not significantly differ. The weights of spleen were 2.1 \pm 0.1 [FeSO₄ (200 mg)], 0.6 \pm 0.0 [β -CN (1–25)(200 mg)], 1.2 \pm 0.1 (pair-fed), and 1.1 \pm 0.1 (Cas-Fe); these four groups were different (ANOVA, p < 0.05): FeSO₄ (200 mg) was higher than the three other groups (Fisher's exact t test, p < 0.05); β -CN (1–25) (200 mg) was lower than pair-fed and FeSO₄ (200 mg) groups (p < 0.05).

Because of the differences in the weight of organs, Fe concentrations of liver and spleen are shown in Figure 1 and their total content in Figure 2.

DISCUSSION

Digestive interactions between proteins and Fe are not fully defined: increased Fe dialyzability following in vivo digestion of various kinds of proteins appears to be more predictive of its absorption rate than its solubility (Hurrell et al., 1988; Kane and Miller, 1984;

Table 2. Changes in Hemoglobin Concentration and Red Blood Cell Count (RBC) during Repletion of Weaning Rats (4 Weeks Deficiency, 2 Weeks Repletion) Fed either FeSO₄, ss-CN (1–25) Bound Fe, or ss-Casein Bound Fe and Control, Nondeficient Rats

	hemoglobin				RBC			
	beginning of repletion	end of repletion	change	ANOVA, $p < 0.001$	beginning of repletion	end of repletion	change	ANOVA, $p < 0.001$
(A) Fe Supply during Repletion = 40 mg of Fe/kg of Diet								
FeSO ₄	5.7 ± 0.8 ^a	11.9 ± 1.6	6.2 ± 1.7	$p < 0.05$	3.39 ± 0.11	7.23 ± 0.39	3.84 ± 1.24	$p < 0.05$
(1–25)	5.3 ± 0.1	11.6 ± 1.1	6.3 ± 1.2	$p < 0.05$	3.21 ± 0.05	6.74 ± 0.18	3.66 ± 0.25	$p < 0.05$
ANOVA	$p = 0.33$	$p = 0.71$	$p = 0.50$		$p = 0.32$	$p = 0.39$	$p = 0.56$	
(B) Fe Supply during Repletion = 200 mg of Fe/kg of Diet								
pair-fed	15.5 ± 0.8 ^a	15.1 ± 1.0	-0.6 ± 1.6	NS	7.07 ± 0.25	7.41 ± 0.16	0.15 ± 0.67	NS
FeSO ₄	5.6 ± 1.0 ^a	13.2 ± 0.9 ^{ab}	7.6 ± 0.9 ^{ab}	$p < 0.05$	3.47 ± 0.25 ^a	6.23 ± 0.15 ^{ac}	2.75 ± 0.47 ^{ac}	$p < 0.05$
(1–25)	6.3 ± 0.2 ^a	15.1 ± 0.3	8.8 ± 0.7 ^a	$p < 0.05$	3.77 ± 0.13 ^a	6.50 ± 0.05 ^a	2.72 ± 0.30 ^a	$p < 0.05$
Cas-Fe	5.4 ± 0.1 ^{ab}	13.6 ± 0.7 ^{ab}	8.2 ± 0.8 ^a	$p < 0.05$	3.31 ± 0.05 ^a	6.93 ± 0.11 ^b	3.63 ± 0.36 ^{ab}	$p < 0.05$
ANOVA	$p < 0.001$	$p < 0.001$	$p < 0.001$		$p < 0.001$	$p < 0.001$	$p < 0.001$	

^a Hemoglobin, g/dL; RBC, 10⁶/mL; $x \pm SEM$; $n = 8$ /group. Source of iron: FeSO₄, FeSO₄·7H₂O; (1–25), Fe bound to β -CN (1–25) of β -casein; Cas-Fe, Fe bound to whole β -casein; pair-fed, nondeficient control rats pair-fed to experimental groups (6 weeks, 200 mg/kg FeSO₄). Statistical analysis, ANOVA with repeated measures followed by Fisher's exact t test: within rows, effect of time; within columns, differences between groups, Fisher's exact t test (a, vs pair-fed; b, vs 1–25 CPP; c, vs Cas-Fe; $p < 0.05$).

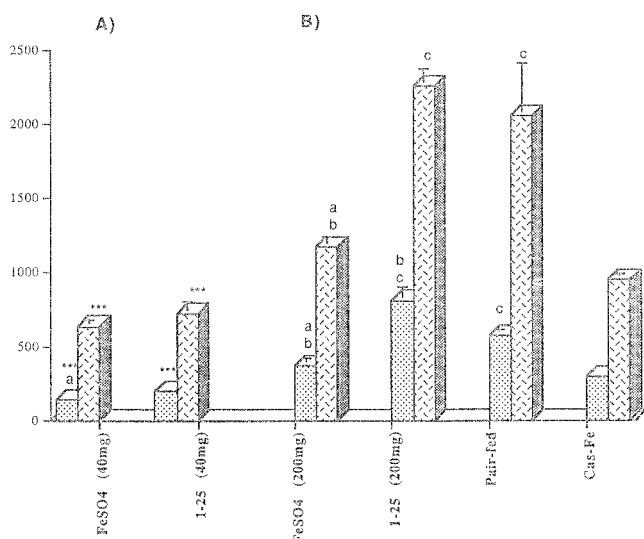


Figure 1. Liver and spleen Fe concentrations after repletion of Fe-deficient weaning rats (4 weeks deficiency, 2 weeks repletion) fed either FeSO₄ or β -CN (1–25) bound Fe: (A) repletion with 40 mg of Fe/kg of diet; (B) repletion with 200 mg of Fe/kg of diet; (dotted bars) liver iron (μ g/g); (cross-hatched bars) spleen iron (μ g/g); (*) μ g/g; $x \pm SEM$; $n = 8$ /group. Source of iron: FeSO₄, FeSO₄·7H₂O; (1–25), Fe bound to β -CN (1–25) of β -casein; Cas-Fe, Fe bound to whole β -casein; pair-fed, nondeficient control rats pair-fed to experimental groups (6 weeks, 200 mg/kg FeSO₄). Influence of the Fe source: (ANOVA followed by Fisher's exact t test) a, different ($p < 0.05$) from (1–25); b, different ($p < 0.05$) from pair-fed; c, different from Cas-Fe ($p < 0.05$). Liver: (A) ANOVA, $p = 0.007$; (B) ANOVA, $p < 0.001$. Spleen: (A) ANOVA, no difference between groups; (B) ANOVA, $p < 0.001$. A vs B: (***) different from 200 mg group fed the same form of Fe (Fisher's exact t test; $p < 0.05$).

Kim et al., 1995; Miller and Berner, 1989); thus, a protecting effect of proteins does not rely only on their ability to keep Fe in a reduced and soluble form but depends also on the weight of the complexes that can be formed: high molecular weight proteins could be excluded from the brush border surface by the mucus, preventing access of Fe to membrane receptors (Miller and Berner, 1989).

The strong affinity of egg and milk phosphoproteins for divalent cations (Baumy and Brulé, 1988; Brulé and Fauquant, 1982; Hegenauer et al., 1979; West, 1986) is resistant to phosphatases (Bouhallab et al., 1991). When ingested in an intact form, these proteins are assumed

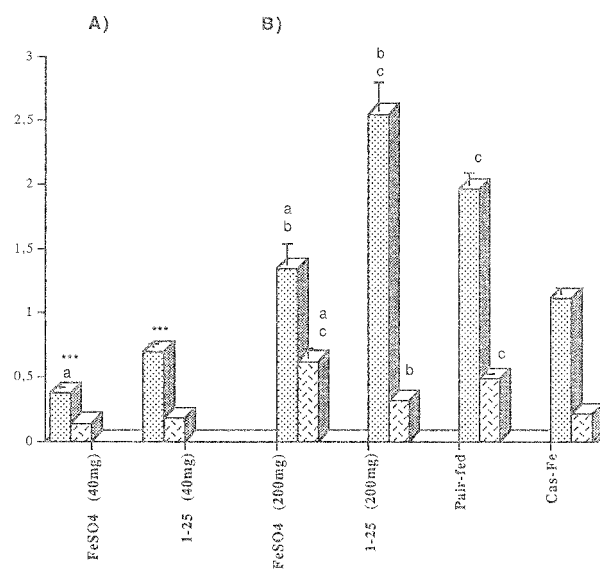


Figure 2. Fe total content of liver and spleen after repletion of Fe-deficient weaning rats (4 weeks deficiency, 2 weeks repletion) fed either FeSO₄ or β -CN (1–25) bound Fe: (A) repletion with 40 mg of Fe/kg of diet; (B) repletion with 200 mg of Fe/kg of diet; (dotted bars) liver iron (μ g/g); (cross-hatched bars) spleen iron (μ g/g); (*) mg; $x \pm SEM$, $n = 8$ /group. Source of iron: FeSO₄, FeSO₄·7H₂O; (1–25), Fe bound to β -CN (1–25) of β -casein; Cas-Fe, Fe bound to whole β -casein; pair-fed, nondeficient control rats pair-fed to experimental groups (6 weeks, 200 mg/kg FeSO₄). Influence of the Fe source: (ANOVA followed by Fisher's exact t test) a, different ($p < 0.05$) from (1–25); b, different ($p < 0.05$) from pair-fed; c, different from Cas-Fe ($p < 0.05$). Liver: (A) ANOVA, $p = 0.001$; (B) ANOVA, $p < 0.001$. Spleen: (A) ANOVA, no difference between groups; (B) ANOVA, $p < 0.001$. A vs B: (***) different from 200 mg group fed the same form of Fe (Fisher's exact t test; $p < 0.05$).

to decrease Fe bioavailability (Morris, 1983; Wapnir, 1990; West, 1986) yet they bind and keep it soluble at luminal pH (Baumy and Brulé, 1988; Galdi and Valencía, 1988; Hegenauer et al., 1979); hydrolyzing them into low molecular weight peptides prior to their ingestion improves Fe absorption, as previously shown in human studies (Hurrell et al., 1989).

The 25 amino acid phosphopeptide of β -casein [β -CN (1–25)] forms soluble and steady complexes with Fe at luminal pH (Meisel and Frister, 1989; Bouhallab et al., 1991) and enhances Fe absorption by isolated duodenal loop (Péres et al., 1997, 1998).

The results of the present study support the good bioavailability of β -CN (1–25) bound Fe: it improved both Fe tissue storage and Hb levels during repletion of the deficient young rat, as compared to free FeSO₄; Fe bound to the whole, nonhydrolyzed casein had a similar effect on Hb levels but was less efficient in improving tissue stores. Total liver Fe levels reflect the size of Fe stores such as ferritin Fe and are similarly depleted during Fe deficiency (Siimes et al., 1980; Dhur et al., 1989).

Improved Fe luminal solubility could have contributed to the availability of β -casein Fe to restore Hb levels; the better efficiency of β -CN (1–25) bound Fe, which enhanced also Fe storage by organs, seems to support the hypothesis of a barrier effect of mucus against large peptides (Miller and Berner, 1989). The observation by Kasai et al. (1995) of a similar content of CPP in the ileum of rats fed either casein or CPP does not take into account the kinetics of protein hydrolysis that yields digestion products throughout the upper part of the digestive tract, whereas Fe absorption can occur only in the duodenum and proximal jejunum (Beard, 1996); therefore, it appears useful that Fe reaches the upper part of the bowel yet bound to low molecular weight ligands which could favor its absorption.

In addition, CPP-bound Fe could be absorbed through a different mechanism than inorganic Fe: part of β -CN (1–25) bound Fe is absorbed by endocytosis (Pérès et al., 1999); enterocytes are able to uptake large peptides, up to 30 kDa (Pantzar et al. 1993); uptake and plasma detection of biologically active peptides derived from caseins have also been consistently reported (Maubois and Léonil, 1989; Chabance et al., 1998). These facts support the assumption that at least part of β -CN (1–25) bound Fe could pass into the blood still in a bound form.

Supplying Fe in a bound form could also influence its metabolism in the organism once absorbed and explain its improved tissue storage as compared to inorganic Fe: as shown for zinc, the presence of amino acids and peptides determines the metabolism of trace elements, in addition to influencing their absorption. Adding milk proteins to a zinc-containing meal improves its absorption yet it decreases its serum level, suggesting an enhanced tissue clearance of absorbed metal (Sandström et al., 1980).

The deficient rat has been used extensively to evaluate the bioavailability of Fe compounds used or proposed for fortification of human food (Mahoney and Hendricks, 1984): it has a reduced sensitivity to dietary effects and cannot be used to quantify the influence of these factors in human nutrition (Reddy and Cook, 1991). However, the low sensitivity of this model suggests that differences in the efficiency of the two forms of Fe in restoring Fe stores and Hb levels which are displayed in the rat would still occur in humans; this should be demonstrated in further clinical studies.

CONCLUSION

Binding Fe to the 1–25 caseinophosphopeptide issued from the enzymatic hydrolysis of β -casein led to a better correction of anemia than FeSO₄ and improved Fe stores better than FeSO₄ and whole casein bound Fe. The dietary source of CPP and the protecting effect against peroxidation of complexes of Fe-bound phosphopeptides (Hegenauer et al., 1979) suggest they have a potential interest in food fortification.

ABBREVIATIONS USED

CPP, caseinophosphopeptides; β -CN (1–25), 1–25 caseinophosphopeptide of β -casein; FeSO₄ 40 mg or FeSO₄ 200 mg, animals repleted with Fe²⁺SO₄ at a concentration of 40 mg or 200 mg of Fe/kg of diet; β -CN (1–25) 40 mg, β -CN (1–25) 200 mg, animals repleted with β -CN (1–25) bound Fe at a concentration of 40 mg or 200 mg of Fe/kg of diet; Cas-Fe 200 mg, animals repleted with β -casein bound Fe at a concentration of 200 mg of Fe/kg of diet.

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