Effect of calcium-fortified milk-rich diets (either goat's or cow's milk) on copper bioavailability in iron-deficient anemia

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ORIGINAL PAPER

Effect of calcium-fortified milk-rich diets (either goat's or cow's milk) on copper bioavailability in iron-deficient anemia

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Abstract As Cu is a mineral involved in the hematopoietic system whose deficiency is associated with anemia due to its requirement for efficient Fe utilization, the objective of the present study was to assess the effect of fortifying Ca in goat's milk, in comparison to similarly fortified cow's milk. This was performed to check whether Ca-fortified goat's milk minimizes Ca–Cu interactions which would favor Cu bioavailability in experimentally induced iron-deficient (ID) rats. Currently, Ca-enriched dairy products are consumed despite the possibility of mineral interactions such as Ca–Cu. Previous studies have shown that consuming goat's milk improves Cu bioavailability by minimizing Cu–Fe interactions. In the present study, Ca-fortified goat's milk (2× Ca requirement), compared to fortified cow's milk, increased the digestive and metabolic utilization of Cu ($P<0.001$) and Cu content in target organs involved in erythropoiesis (sternum) in ID rats ($P<0.001$). We conclude that goat's milk, even fortified with Ca, could be beneficial for the recovery from iron-deficient anemia by increasing the Cu bioavailability, an essential mineral for erythropoiesis.

摘要 由于铜元素直接参与造血过程，是红血球生成的必需元素，铜的缺乏会使铁的利用率降低因而导致缺铁性贫血。本文比较和评价了强化钙羊奶和牛奶对铜生物利用率的影响。在缺铁性小鼠的模型中研究了钙强化羊奶能够使铜发挥有效生物利用的最低Ca-Cu作用剂量。普遍认为富含钙的乳制品在人体内可能会产生金属元素之间的相互作用，前人的研究结果也证明了羊乳可以将Cu-Fe相互作用程度降低到最小而改善铜的生物利用率。体内试验证明了钙强

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化羊奶 (2倍需要量) 对铜的消化和代谢利用 (P<0.001) 和模型小鼠目标器官 (如红血球生成，胸骨) 中铜含量均高于钙强化牛奶 (P<0.001)。因此，羊奶和钙强化羊奶可以提高铜的生物利用率，有助于缺铁性贫血的治疗。

Keywords Goat's or cow's milk · Ca fortification · Fe-deficient anemia · Cu bioavailability

关键词 羊奶或牛奶 · 钙强化 · 缺铁性贫血 · 铜生物利用率

1 Introduction

Many dairy products are enriched with Ca and are consumed by people of all ages. However, excessive Ca intake may have adverse effects on the metabolism of other micronutrients. In this sense, Ca supplementation can inhibit the absorption of Fe (Barton et al. 1983) perhaps due to previously reported Ca–Cu interactions (Shackelford et al. 1994). Dietary Cu is required for the efficient utilization of dietary Fe. Indications of Fe-deficient anemia can appear within 2 weeks of feeding weaning rats a Cu-deficient diet (Reeves et al. 2004). Cu also acts as a ligand to ferroxidase II, which oxidizes Fe, allowing it to be mobilized and transported from hepatic stores to the bone marrow for use in erythropoiesis (Turnlund 1998). Patients suffering from Cu deficiency can also develop profound hematopoietic deficits resulting in anemia and leucopenia (Gregg et al. 2002). Cu deficiency may inhibit Fe absorption from the gut. Indeed, the Cu-dependent intestinal ferroxidase hephaestin is required for Fe absorption (Chen et al. 2004). Moreover, Cu deficiency has been associated with lower plasma and brain concentrations of Fe, and impaired enterocyte Fe transfer due to decreased hephaestin activity which further lowers plasma Fe and leads to Fe deficiency (Pyatskowit and Prohaska 2008).

Bovine milk and dairy products, which are rich in Ca, interfere with the absorption of Fe from the diet. However, recent studies have shown that when goat's milk is incorporated into the diet, it produces a greater nutritive utilization of Fe in ID animals (Alférez et al. 2006) and minimizes the possible interactions of Fe with other minerals such as Ca, in comparison with animals fed with cow's milk (López Aliaga et al. 2000).

Little is known about Ca-fortified milk rich diets on the Cu bioavailability in iron-deficient anemia (IDA). We hypothesized that Ca-fortified goat's milk consumption, compared to similarly fortified cow's milk, could minimize the Ca–Cu interactions, thus favoring Cu bioavailability. Therefore, the objective of the present study was to determine whether Ca fortification in a goat's milk-based diet in comparison to a cow's milk-based diet has a positive effect on nutritive Cu utilization as well as the distribution of Cu in target organs of rats with IDA.

2 Materials and methods

2.1 Experimental diets

Table 1 summarizes the different diets assessed. The diets were prepared following the recommendations of the AIN-93G formulation (Reeves et al. 1993) except for

Table 1 Composition of experimental diets

	Cow's milk diets (normal or double Ca content)	Goat's milk diets (normal or double Ca content)
Ingredients (g kg^{-1} DM)		
Fat	102.8	103.9
Protein	204.6	202.8
Lactose	172.3	171.8
Wheat starch	331.9	333.4
Constant ingredients	203.1	203.1
Sucrose	100	100
Cellulose	50	50
Mineral mix	35	35
Vitamin mix	10	10
Choline chloride	2.5	2.5
L-Cystine	3.0	3.0
Mineral composition (mg kg^{-1} DM)		
Ca	5,700/10,800[a]	5,800/11,000[a]
Fe	45	46
Cu	5.9	6.0

DM dry matter

[a] Ca content: normal/double in diets. To reach double of Ca requirements, CO_3Ca was added

the total fat content (10% versus 7%) and Ca content in the fortified diets which was double that of the AIN-93G requirement (10 g kg^{-1}). The milk-based diets were made using lyophilized cow's milk (from the Holstein breed) or goat's milk (from the Murciano–Granadina breed) which had been analyzed to determine the composition of fat, protein, lactose, and minerals. The quantities of lyophilized cow's and goat's milk used were taken to obtain diets with a 10% fat content to highlight the possible effects of differences between cow's and goat's milk consumption so that cow's and goat's lyophilates would constitute 33% of the diets. Protein provided by the milk lyophilates alone would be insufficient to support growth. To obtain a total protein content of 20%, the diet was supplemented with cow's milk casein (124 g kg^{-1}) for the cow's milk diet and with goat's milk casein (144 g kg^{-1}) for goat's milk diet. The amounts of copper derived from goat's and cow's milk in the experimental diets were 0.83 and 0.46 mg $kg^{-1}$and the amounts of copper derived from cupric carbonate were (copper source of AIN-93G mineral mix) 5.17 and 5.44 mg kg^{-1} diet, respectively.

2.2 Experimental design

In total, 80 recently weaned male Wistar albino rats were used for this study. The rats were randomly divided into two groups, a control group receiving a modified AIN-93G diet with normal Fe content (45 mg kg^{-1} diet) and an ID group receiving a

modified AIN-93G diet with low Fe content (5 mg kg^{-1} diet) for 40 days. This method of diet-induced IDA was previously validated by our group (Pallarés et al. 1993). After receiving the low-Fe diet for 40 days, peripheral blood samples from the caudal vein were collected for the hematological confirmation of IDA. The rats were defined as anemic as indicated by previously defined parameters (Campos et al. 1998) including low serum Fe (698±88.5 μg L^{-1}), hemoglobin (78.4±2.75 g L^{-1}), red blood cell count ($5.5\pm0.2\times10^{12}$ L^{-1}), hematocrit (27.6±0.6%), and elevated platelets ($1{,}360\pm45.7\times10^{9}$ L^{-1}).

Both the control and the ID groups were fed for 14 days with one of four different diets, cow's and goat's milk diets with either normal Ca content (5 g kg^{-1}) or higher Ca content (10 g kg^{-1}). Animals were housed in individual (Tecniplast® metabolic cages 3700M071; Buguggiate-Va, Italy), ventilated, thermo-regulated cages (22±2 °C) with a 12 h light–dark cycle, 55–60% humidity, and both diet and mineral-free water were provided ad libitum. Body weight was recorded at the beginning and end of the experimental period, and feces and urine were collected separately during the final 7 days of study. On day 15, rats were fasted overnight and anesthetized by intraperitoneal injection of 50 mg kg^{-1} body weight of sodium pentobarbital (Sigma Chemical Co., St Louis, MO, USA). After median laparotomy, rats were bled by cannulation of the abdominal aorta. The spleen, liver, sternum, both femurs, kidneys, and heart were removed, immediately frozen in liquid nitrogen, and stored at −40 °C until analysis. All experiments were undertaken according to the Directional Guides Related to Animal Housing and Care (European Community Council 1986).

2.3 Chemical analysis of diets, feces, urine, and organs

The water content of the diet, feces, liver, sternum, femur, spleen, kidney, and heart was determined by drying the materials at 105±2 °C until the weight remained constant. Diet nitrogen content was determined by the Kjeldahl method using a protein conversion factor of 6.38, and fat content was determined by extraction with petroleum ether (boiling point 40–60 °C) after hydrochloric hydrolysis (Sanderson 1986). Ca, Fe, and Cu were determined in the diet and Cu was also determined in feces, urine, and organs by atomic absorption spectrophotometry using a Perkin-Elmer 1100B spectrophotometer (Norwalk, CT, USA). The samples had previously been mineralized by the wet method in a sand bath (J.R. SELECTA, Barcelona, Spain) with nitric acid, and added perchloric and hydrochloric acids.

2.4 Quality control

Analytical results were validated by comparison to standard reference materials for skim milk powder CRM-063R and lyophilized bovine liver BCR-185 (Community Bureau of Reference Commission of the European Communities, Brussels, Belgium). The mean Ca concentration ± SD of five independent replicates of CRM-063R was 13.49±0.10 μg g^{-1} vs. certified values of 13.35±0.12 μg g^{-1}. Mean Fe concentration ± SD of BCR-185 was 214±5 μg g^{-1} vs. 210±6 μg g^{-1}, and mean Cu concentration was 180±4 μg g^{-1} vs. 187±3 μg g^{-1}.

2.5 Biological indices

The following indices were calculated from the data on Cu intake and fecal and urinary Cu excretion. ADC (apparent digestibility coefficient) $= (I - F) \times 100/I$; R (retention) $= I - (F + U)$; % R/I(retention / intake) $= I - (F + U) \times 100/I$, where I = Cu intake (expressed as dry weight), F = fecal Cu excretion, and U = urinary Cu excretion.

2.6 Statistical analysis

Statistical analysis was performed using the SPSS software (SPSS version 15.0 2007, SPSS Inc. Chicago, IL, USA). Data were analyzed by 2×2×2 factorial model: milk factor (goat's milk or cow's milk), anemia factor (control or ID rats), and calcium concentration factor (normal Ca or double Ca diet) by the least square method (Steel and Torrie 1984). The model accounts for variations caused by these three factors. The second-order interaction between factors was also considered. When the interaction terms were not statistically significant ($P>0.05$), the least square means were calculated from the model after these terms were omitted (Steel and Torrie 1984). Differences were considered significant at $P<0.05$.

3 Results and discussion

3.1 Digestive and metabolic utilization of Cu

When control and ID rats were given the milk-based diets for 14 days with normal or double Ca content, the ADC, balance, and R/I values of Cu were higher with the goat's milk diets compared to the cow's milk diets ($P<0.001$) (Table 2). These findings are in agreement with the higher values of hemoglobin regeneration efficiency obtained with a goat's milk diet as previously reported (Alférez et al. 2006). Moreover, goat's milk has greater medium chain triglyceride (MCT) content than cow's milk (Alférez et al. 2006), and these fatty acids favor the transport of nutrients, including Cu, through the enterocyte basolateral membrane (Tappenden et al. 1997). MCT are absorbed without re-esterification and directly enter portal circulation where they can be metabolized for energy (García-Unciti 1996).

The higher digestive and metabolic utilization of Cu (Table 2, $P<0.001$) detected in the ID compared to the control rats (regardless of milk type and Ca intake) could be because a deficiency of divalent Fe cations in the intestine can increase the absorption of other divalent cations such as Cu (Campos et al. 1998). In a previous study, this effect had been shown in ID rats before supplying the diet with Fe (Rodríguez-Matas et al. 1998). There have been some reports on isolated epithelial cells, suggesting that the main intestinal Fe transporter DMT1 (divalent metal ion transporter) can also transport Cu across the apical membrane (Arredondo et al. 2003; Sharp 2004) and this transporter could be regulated by both Fe and Cu (Arredondo et al. 2003). Extended periods of iron deficiency could lead to an up-regulation of DMT1 expression which subsequently produces an increase in Cu absorption in ID rats. In addition, Gómez-Ayala et al. (1998) showed that in

Table 2 Digestive and metabolic utilization of Cu in control and ID groups fed diets with normal or double Ca content

	Cow's milk diet				Goat's milk diet				Diet	Fe deficiency	Ca supplement
	Control mean SD n=20		ID mean SD n=20		Control mean SD n=20		ID mean SD n=20				
Cu intake (μg day^{-1})											
Normal Ca	91.3	4.1	95.4	5.0	84.8	6.1	87.3	5.2	0.32	0.45	0.09
Double Ca	89.6	4.2	95.0	5.1	89.3	5.0	88.2	6.2			
Fecal Cu (μg day^{-1})											
Normal Ca	59.5	6.7a	56.6	7.1A	42.7	4.9b	35.5	3.7B	0.0012	0.03	0.25
Double Ca	59.9	7.1a	61.2	7.4A	48.1	5.1b	41.4	4.8B			
ADC (%)											
Normal Ca	34.6	1.2a	40.6	1.1Ac	49.5	2.0b	57.2	3.1Bc	0.0001	0.0014	0.15
Double Ca	32.9	1.0a	37.1	0.9AcC	46.1	1.4b	53.0	2.1Bc			
Urinary Cu (μg day^{-1})											
Normal Ca	4.6	0.05	4.2	0.10	3.5	0.04	3.4	0.01	0.46	0.75	0.64
Double Ca	4.1	0.02	3.8	0.07	3.4	0.03	3.7	0.05			
Cu retention (μg day^{-1})											
Normal Ca	27.1	2.1a	34.6	3.1Ac	38.7	5.4b	48.4	6.2B	0.0002	0.0013	0.56
Double Ca	25.5	2.7a	32.0	2.1Ac	37.8	4.1b	43.1	5.9B			
R/I (%)											
Normal Ca	29.5	1.5a	36.3	1.0Ac	46.9	3.0b	55.4	3.7Bc	0.0014	0.0019	0.03
Double Ca	28.2	1.0a	33.2	0.9AcC	43.3	2.2b	49.8	2.8Bc			

n=20 (normal Ca=10, double Ca=10). Interactions: diet × supplement: $P<0.05$ for ADC, retention, and *R/I*; diet × Fe deficiency × supplement: $P<0.05$ for ADC and *R/I*

ID iron deficient, *SD* standard deviation, *NS* not significant ($P>0.05$), *ADC* apparent digestibility coefficient, *R/I* retention/intake, *Diet* main effect of diet, *Fe deficiency* main effect of Fe deficiency, *Ca supplement* main effect of Ca supplement. *a*, *b* Within the same row, values of control groups with different letters are different ($P<0.05$). *A*, *B* Within the same row, values of Fe-deficient groups with different letters are different ($P<0.05$). *c* Values were significantly different from the corresponding control group ($P<0.05$). *C* Values were significantly different from the corresponding normal Ca group ($P<0.05$)

IDA, Cu absorption increases. However, the main Cu transporter regulating Cu uptake into the enterocyte is Ctr1 (Zimnicka et al. 2007). This Cu transporter is specific for Cu, and Fe is therefore not expected to interfere with Cu absorption, so Ctr1 could not be involved in the increase in Cu absorption in ID rats. On the other hand, Domellöf et al. (2009) found that Fe supplementation does not affect Cu absorption in breastfed infants.

Adequate dietary Cu and Cu absorption is required for efficient absorption and utilization of dietary Fe in rats. Reeves and DeMars (2004) found that Cu-deficient rats retained less dietary ^{59}Fe than Cu-adequate rats using whole-body ^{59}Fe counting. Cu facilitates intestinal Fe absorption by promoting Cu-dependent ferroxidase (hephaestin) activity in the duodenal enterocyte. Signs of Fe deficiency

such as low serum Fe and anemia appear in weaning rats within a few days of consuming a Cu-deficient diet (Reeves et al. 2004). This finding suggests a very important role for Cu in Fe absorption. In addition, Cu affects the Cu-dependent ferroxidase activity of ceruloplasmin, a plasma enzyme that catalyzes the oxidation of ferrous ion into the ferric ion required for hemoglobin synthesis. Afterwards, hemoglobin is transported from hepatic stores to the bone marrow to be used in erythropoiesis (Turnlund 1998). Cu-deficient rats are anemic and ceruloplasmin activity is reduced to near zero (Reeves and DeMars 2004).

In ID rats fed on the cow's milk diet, there was a negative effect of Ca fortification on ADC ($P<0.01$) and R/I ($P<0.05$) of Cu, but there was no such influence with the goat's milk diet (Table 2). These findings could be due to the nutritional characteristics of the goat's milk (Barrionuevo et al. 2002) minimizing Ca–Cu interactions (Shackelford et al. 1994), even though the dietary Ca content was double that required for the rat.

4 Tissue Cu concentration

Comparison of the milk diets shows that the Cu content in sternum, kidney, spleen, and heart was higher for both the control and ID groups fed on the goat's milk diet compared to the cow's milk diet ($P<0.001$). Moreover, Cu in the kidney was two-fold higher when ID rats were given the Ca-fortified goat's milk ($P<0.001$), as compared to a cow's milk diet (Table 3). Goat's milk diets, with both normal Ca and elevated Ca, favored higher Cu deposition in the sternum, kidney, spleen, and heart in comparison to the cow's milk diet. This fact is probably a consequence of higher Cu absorption and retention as indicated above. For all groups, the highest concentration of Cu was found in the kidneys. This result is in agreement with a report by Linder (1996) who showed that Cu distribution throughout the organism is accomplished in two phases. The first phase involves the transfer of Cu from the intestine to the liver and the kidneys, and the second phase involves distribution from the liver to the other organs. The kidney is rich in Cu, and as has been shown by Barrionuevo et al. (2002) this organ consistently reflects Cu absorption and metabolism. However, in this study the renal copper concentration did not always reflect Cu absorption and metabolism.

Ca fortification with an 11 g Ca kg^{-1} diet had a negative effect on the Cu content in the femur, sternum, and kidney for the ID animals fed on the cow's milk diet ($P<0.001$) and no differences in Cu concentrations were detected in the spleen, liver, and the heart (Table 3). However, it is noteworthy that Ca fortification significantly raised the quantity of Cu deposited in the sternum and spleen ($P<0.01$), whereas copper deposits in the femur and liver remained unchanged in the ID rats given the goat's milk diet. These results are in agreement with the improved Cu R/I ratio with the goat's milk diet (with both normal Ca and fortified Ca diets) in comparison to cow's milk diet. Whereas goat's milk diet fortified with Ca raised the quantity of Cu deposited in the sternum in the ID group, the contrary effect was observed with the cow's milk diet; it can be related with the negative effect of Ca fortification on Cu retention with the cow's milk diet, whereas with the goat's milk diet Cu retention is not affected.

Table 3 Cu concentrations in several organs in control and ID groups fed diets with normal or double Ca content

(μg g^{-1} DM)	Cow's milk diet				Goat's milk diet				Diet	Fe deficiency	Ca supplement
	Control mean	SD	ID mean	SD	Control mean	SD	ID mean	SD			
	n=20		n=20		n=20		n=20				
Femur											
Normal Ca	6.2	0.41	6.6	0.42	6.1	0.29	6.1	0.47	0.15	0.04	0.03
Double Ca	6.0	0.49	5.5	0.13C	5.6	0.17	6.1	0.14c			
Sternum											
Normal Ca	7.3	0.57	10.0	0.51c	8.4	0.35	10.3	0.41c	0.0019	0.009	0.04
Double Ca	8.0	0.69	8.9	0.24AC	9.0	1.75	12.1	0.95BC			
Kidney											
Normal Ca	48.6	1.1a	37.7	3.0Ac	55.1	2.2b	60.5	1.9Bc	0.0002	0.0013	0.0011
Double Ca	41.7	2.1aC	27.3	1.5AcC	49.7	1.7bC	53.5	1.0BcC			
Spleen											
Normal Ca	8.4	0.61a	8.9	0.42A	11.0	1.11b	12.6	0.75B	0.0009	0.55	0.02
Double Ca	9.8	0.75a	10.3	0.97A	13.2	1.03b	14.3	0.41BC			
Liver											
Normal Ca	14.3	1.4	16.3	1.9	14.9	1.4	16.6	1.8	0.63	0.73	0.56
Double Ca	13.5	1.2	16.4	1.8	14.6	1.3	15.0	1.5			
Heart											
Normal Ca	17.1	1.3	17.4	1.2A	18.1	1.4	24.2	1.5Bc	0.0019	0.03	0.02
Double Ca	18.3	1.1	16.1	0.2c	18.3	1.7	20.6	0.5C			

n=20 (normal Ca=10, double Ca=10). Interactions diet × Fe deficiency, diet × supplement, and Fe deficiency × supplement: P<0.01 for kidney

ID iron deficient, *DM* dry matter, *SD* standard deviation, *NS* not significant (P>0.05), *Diet* main effect of diet, *Fe deficiency* main effect of Fe deficiency, *Ca supplement* main effect of Ca supplement. *a*, *b* Within the same row, values of control groups with different letters are different (P<0.05). *A*, *B* Within the same row, values of Fe-deficient groups with different letters are different (P<0.05). *c* Values were significantly different from the corresponding control group (P<0.05). *C* Values were significantly different from the corresponding normal Ca group (P<0.05)

Moreover, this finding of elevated Cu in the sternum and spleen may be very important because this is a required mineral in the formation of erythrocytes (Turnlund 1998), and these are important organs in the erythrocyte life cycle.

5 Conclusion

The consumption of goat's milk fortified or not with Ca in rats with IDA minimizes Ca–Cu interactions due to an increase in the digestive and metabolic utilization of Cu and its deposition in the spleen and sternum, the latter being a key organ in erythropoiesis. From a public health perspective, the consumption of goat's milk, fortified or not with Ca, could be included in the diet in

individuals suffering with IDA due to the positive effects on Cu metabolism. However, further studies must be performed to check if these positive effects could be extrapolated to humans.

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