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Effect of calcium-supplemented goat or cow milk on zinc status in rats with nutritional ferropenic anaemia

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ABSTRACT

The effects of goat- or cow-milk-based diets, whether normal or supplemented with calcium (5 or 10 g kg^{-1} Ca, respectively), on the nutritive utilization of Zn and its deposition in organs, were evaluated in rats with and without nutritional ferropenic anaemia (NFA). The digestive and metabolic utilization of Zn in anaemic and control groups was higher for rats on the goat milk diet than those on the cow milk diet. The most noteworthy result is that Ca supplementation in the diet improved Zn metabolism in all experimental groups, but especially in anaemic rats fed the goat milk diet. This fact is reflected in the higher levels of Zn deposition found with the goat milk diet. It appears that goat milk, especially when supplemented with Ca, had beneficial effects on nutritive utilization of Zn and Zn deposition in target organs in both control rats and, especially, rats with NFA.

1. Introduction

Zinc is the most abundant intracellular trace element in animals and plays a structural role in metalloproteins, such as the cytosolic enzyme CuZn superoxide-dismutase (Hambidge et al., 1986). Most studies in this field have shown that subjects with moderate Zn deficiency have restricted linear growth, which is reversible with Zn supplementation. Moreover, moderate Zn deficiency causes an increase in respiratory, digestive and skin diseases by diminishing the immune response, and evidence exists that relates Zn deficiency with cognitive and motor function in children (Black, 2003a, 2003b; Castillo Duran & Weisstaub, 2003).

As we previously reported, compared with cow milk, goat milk consumption improves Zn bioavailability (Alférez et al., 2003), but no information is currently available on the influence of different milk sources on the metabolism of Zn in a state of nutritional ferropenic anaemia (NFA). This pathology causes haematological and hormonal changes, such as higher levels of cortisol and parathormone (PTH) in serum (Campos et al., 1998), stunted growth, altered thermoregulatory function and decreased cognitive function. NFA is considered to be a major public health problem and the most common nutritional deficiency in the world (Viteri, 1993).

Nowadays, many dairy products are supplemented with Ca. These products are consumed by people of all ages, independent of their health status. Increased Ca supplementation may have an adverse effect on the metabolism of other micronutrients. Wood and Zheng (1997) reported that Ca intake at 1360 mg day^{-1} decreased the apparent absorption and retention of Zn in humans. Dursun and Aydogan (1994) showed that a diet containing 20 g Ca kg^{-1} decreased the true absorption of Zn in rats. Takasugi et al., (2007) showed that excess Ca of 25 g kg^{-1} diet, did not affect the true absorption of Zn and its endogenous excretion.

The aim of the present study was to investigate the effects of goat- and cow-milk-based diets, whether normal or supplemented with Ca (5 or 10 g kg^{-1}, respectively), on the nutritive utilization of Zn and its deposit in target organs in control rats and rats with NFA.

2. Materials and methods

2.1. Animals

Male Wistar albino breed rats ($n = 80$) recently weaned, aged about 3 weeks, purchased from the University of Granada Laboratory Animal Service, were used for this study. Animal care procedures and experimental protocols were approved by the Ethics Committee of the University of Granada in accordance with the European Community guidelines.

2.2. Model of nutritional ferropenic anaemia

The rats were randomly divided into two groups: a control group receiving the diet AIN 93 G with a normal-Fe level (45 mg Fe kg^{-1} diet) and an anaemic group receiving the diet AIN 93 G with a low Fe level (5 mg Fe kg^{-1} diet) for 40 days (Fig. 1). Dietary Fe deficiency was induced by a technique previously developed by our

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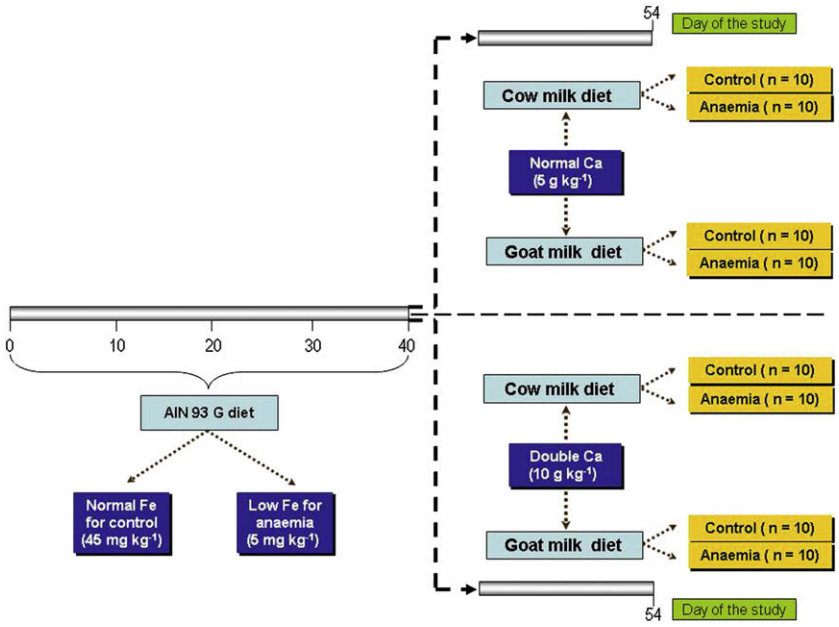

Fig. 1. Experimental design of the study. Rats were randomly divided into two groups: a control group ($n = 40$) receiving the diet AIN 93 G with a normal-Fe level (45 mg Fe kg^{-1} diet) and an anaemic group ($n = 40$) receiving the diet AIN 93 G with a low Fe level (5 mg Fe kg^{-1} diet) for 40 days. On day 40, blood aliquots were obtained from the caudal vein for the measurement of haematological parameters. Both the control and the anaemic groups were then fed for a further 14 days with different types of diets: cow and goat milk diets with a normal Ca content (5 g kg^{-1}) or double the Ca content (10 g kg^{-1}). Food intake was measured and urine and faeces were collected daily for balance studies. On day 15, the rats were fasted overnight and then anaesthetized. After median laparotomy, the rats were totally bled by cannulation of the abdominal aorta and the organs were removed for study.

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 haematological study of the NFA (Table 1).

2.3. Experimental design and diets

The experimental design is presented in Fig. 1. Both the control and the anaemic groups were fed for 14 days with different types of diets: cow and goat milk diets with a normal-Fe content (45 mg kg^{-1}) and either a normal Ca content (5 g kg^{-1}) or a double Ca content (10 g kg^{-1}). From the start of the study, the animals were housed in individual, ventilated, thermoregulated cages (22 ± 2 °C) with a 12 h light–dark period, 55–60% humidity and both the feed and mineral-free water were available ad libitum. During the 14 day experimental period, food intake was measured daily, and urine and faeces were collected daily. Body weight was recorded at the beginning and end of experimental period. On day 15, the rats were fasted overnight and then anaesthetized by intraperitoneal injection of 5 mg 100 g^{-1} body weight of sodium pentobarbital (Sigma Chemical Co, St. Louis, MI, USA). The spleen, liver, sternum, both femurs, kidneys, heart, testes and the brain were removed, frozen immediately in liquid nitrogen and then stored at −40 °C for later Zn analysis.

Table 2 summarizes the different diets assayed. The diets were prepared following the recommendations of the AIN 93 G (Reeves et al., 1993), except for the source and level of fat (10% rather than 7%) and Ca level in Ca supplemented diets (double of the requirement for rats). The milk-based diets were made up using lyophilized cow or goat milk, which were analyzed to determine the fat, protein, lactose content, and mineral composition (Table 2). The necessary quantities of lyophilized cow milk were taken to obtain a diet with a 10% fat content and they constituted a third part of the diets. To obtain a protein content of 20%, the diet was supplemented with cow milk casein for the cow milk diet and with goat milk casein for goat milk diet, as the protein provided by the lyophylate used for the milk-based diets was insufficient.

Table1
Haematological parameters[a] in rats fed for 40 days with either normal-Fe or low-Fe diet

Parameters[b]	Experimental diets	
	Normal-Fe diet: control group	Low-Fe diet: anaemic group
RBC (10^{12} L^{-1})	7.1 ± 0.1	6.5 ± 0.2[c]
MCV (fL)	55.1 ± 0.2	39.4 ± 0.7[c]
Haematocrit (%)	38.6 ± 0.8	27.6 ± 0.5[c]
Platelets (10^9 L^{-1})	738 ± 25	1360 ± 67[c]
Haemoglobin (g L^{-1})	129 ± 2.8	78.4 ± 2.6[c]
Serum iron (μg L^{-1})	1392 ± 123	698 ± 56[c]
Serum ferritin (μg L^{-1})	82.3 ± 2.7	50.2 ± 1.3[c]
Transferrin saturation (%)	47.3 ± 7.2	3.7 ± 0.3[c]
TIBC (μg L^{-1})	2837 ± 205	17915 ± 733[c]

[a] Values are means ± standard error ($n = 40$).
[b] Abbreviations: RBC, red blood cell count; MCV, mean corpuscular volume; TIBC, total iron-binding capacity.
[c] Mean values were significantly different from the corresponding group of the control animals at $P < 0.05$ by Student's t test.

2.4. Analytical methods and biological indices

All reagents were of analytical grade, and ultrapure water of 18 MΩ cm specific resistivity was obtained from a Milli-Q purification system (Millipore Corp., MA, USA).

The water contents of the diet, faeces, liver, sternum, femur, spleen, kidney, heart, testes and brain were determined by drying the materials at 105 ± 2 °C until the weight remained constant.

2.4.1. Zn determination

The samples were previously mineralized by a wet method in a sand bath (J.R. Selecta, Barcelona, Spain) placing the samples into a resistant flask and dissolving using nitric acid, followed by creating a mixture with HNO_3:$HClO_4$ (1:4, v/v) until the total elimination of organic matter. Finally, the samples were diluted with Milli-Q water and filtered with filter Whatman no. 41, to obtain an adequate volume. The concentration of Zn in the diets, faeces, urine and all the

Table 2
Composition of the experimental diets

Component (g kg^{-1})	Amount
(*Cow milk diet* (*normal or double Ca*)[a]	
Lyophilized cow milk[b]	348
Cow milk fat	100
Lactose and minerals	172
Cow milk protein	76
Cow milk casein	124
Wheat starch	329
Constant ingredients[c]	199
Goat milk diet (*normal or double Ca*)[a]	
Lyophilized goat milk[b]	326
Goat milk fat	100
Lactose and minerals	171
Goat milk protein	55
Goat milk casein	145
Wheat starch	330
Constant ingredients[c]	199

[a] Diets contain either normal Ca amount, as recommended by the AIN (1993), or double the requirements. The Ca and P for normal Ca diets were provided by lyophilized milk and the added casein. To reach double the Ca requirement, $CaCO_3$ was added. All diets were supplemented to be adequate in Fe (45 mg kg^{-1}) and isocaloric (17 226 kJ kg^{-1}).

[b] Lyophilized milks were analyzed to determine fat (cow: 28.76%; goat: 30.69%), protein (cow: 24.84%; goat: 23.36%), lactose (cow: 40.75%; goat: 39.20%), and mineral composition (g kg^{-1} of lyophylate: cow: Ca, 10.3; P, 7.8; Mg, 0.85; Fe, 0.087; Cu, 0.0014; Zn, 0.035; goat: Ca, 13.2; P, 8.1; Mg, 0.89; Fe, 0.012; Cu, 0.0025; Zn, 0.0041). In diets with normal Ca levels, the mineral content was (in g kg^{-1} of diet) for cow milk diet: Ca, 5.7; P, 4.0; Zn, 0.038 and for goat milk diet: Ca, 5.8; P, 4.0; Zn, 0.039. In diets with double the Ca level the mineral content was (in g kg^{-1} of diet) for cow milk diet: Ca, 10.8; P, 4.1; Zn, 0.037; and for goat milk diet: Ca, 11.0; P, 4.1; Zn, 0.038.

[c] The constant ingredients consisted of (in g kg^{-1} diet): fibre (micronized cellulose), 50; sucrose, 100; choline chloride, 2.5; L-cystine, 1.8; mineral premix, 35; vitamin premix, 10. The specific mineral premix for cow and goat milk diets were formulated taking into account the mineral content of the diet components supplied in order to meet the recommendations of AINGS93.

organs studied was determined by atomic absorption spectrophotometry (Perkin–Elmer 1100B, Norwalk, CT, USA).

Given the importance of obtaining an accurate determination of the different parameters studied, a quality control test of these determinations was carried out. This consisted in analyzing a lyophilized bovine liver (certified reference material BCR 185; Community Bureau of References, Brussels, Belgium), which yielded a Zn value of 139 ± 2 mg kg^{-1} (mean $\pm$ SEM values of five determinations; certified value: Zn, 143 ± 4 mg kg^{-1}). All glassware and polyethylene sample bottles used were washed using 10 M nitric acid, and Milli-Q water.

The following biological indices were calculated from the data on Zn intake and faecal and urinary Zn excretion:

$$\text{Apparent digestibility coefficient (ADC)} = (\text{intake} - \text{faecal excretion}) \times 100\ \text{intake}^{-1}$$

$$\text{Balance} = (\text{intake} - \text{faecal excretion}) - \text{urinary excretion}$$

$$R/I\ (\%) = \text{balance} \times 100\ \text{intake}^{-1}$$

2.4.2. Nitrogen content

Nitrogen content in the lyophylates and diets was determined by Kjeldahl's method, using a protein conversion factor of 6.38 (FAO/WHO, 2003).

2.4.3. Fat content

Fat content in the lyophylates and diets was determined after hydrochloric hydrolysis by extraction with petroleum ether, boiling point: 40–60 °C (Sanderson, 1986).

2.4.4. Fe determinations

Total iron-binding capacity (TIBC) and serum Fe levels were determined colorimetrically and enzymatically, using Sigma Diagnostics Iron and TIBC reagents (Sigma Diagnostics, St. Louis, MI, USA). Percent transferrin saturation was subsequently calculated using the following equation:

$$\text{Transferrin saturation}\ (\%) = [\text{serum iron concentration}\ (\mu g\ L^{-1})\ /\text{TIBC}\ (\mu g\ L^{-1})] \times 100$$

2.5. Statistical analysis

Statistical analyses were performed using the SPSS computer program (SPSS, version 15.0, 2007; SPSS Inc., Chicago, IL, USA). The data from four trials using the control and anaemic rats were analyzed by the use of factorial arrangement, 2(type of diet: goat versus cow milk diet) $\times$ 2(group of animal: control versus anaemic) $\times$ 2(Ca content in the diet: normal or double), and by the least-squares method (Steel & Torrie, 1984). The model accounts for variations caused by the type of diet, anaemia, Ca supplement and by the interactions between these. When the interaction terms were not statistically significant ($P > 0.05$), the least-squares means were calculated from the model after these terms were omitted (Steel & Torrie, 1984). Differences between groups (control versus anaemic and normal Ca versus double Ca) were tested for statistical significance with Student's t test. Variance analysis by one-way methods was used to compare the different diets supplied to the two groups of animals (control and anaemic). Individual means were tested by pairwise comparison with Tukey's multiple comparison test when main effects and interactions were significant. Differences were considered significant at $P < 0.05$.

3. Results and discussion

After supplying the low-Fe diet during 40 days, the rats were anaemic, as indicated by the low levels of serum Fe, haemoglobin (Hb), red blood cell count (RBC), mean corpuscular volume (MCV), haematocrit, serum ferritin and transferrin saturation ($P < 0.001$), and high levels of platelets and total Fe-binding capacity (TIBC) ($P < 0.001$), consistent with Fe-deficiency-induced anaemia in rats (Table 1).

3.1. Digestive and metabolic utilization of Zn

The effects of type of diet revealed that for the control and the anaemic rats, the apparent digestibility coefficient (ADC), balance and R/I were greater when the animals were given the goat milk diets compared to cow milk diets ($P < 0.001$) (Table 3). The bioavailability of Zn in goat milk is higher than in cow milk, for various reasons. Firstly, goat milk contains a greater amount of vitamins C and D (Alférez et al., 2006) and, according to Hartiti et al. (1994), this could contribute to the higher ADC of Zn in animals fed the goat milk diets. The positive effect of vitamin D might be explained by an increase in the concentration of Zn-binding-protein (CRIP), which binds Zn during transmucosal transport, and this protein may function as an intracellular Zn carrier (Hempe & Cousins, 1992). Vitamin C has relatively little influence (in comparison with Vitamin D) in Zn absorption (Sandstrom & Cederblad, 1987). Furthermore, goat milk is richer in cysteine than is cow milk (Alférez et al., 2006; Souci et al., 1989), this amino acid is active in the absorption and metabolism of Zn (Hempe & Cousins, 1991). Moreover, goat milk has a greater medium chain triglyceride content than does cow milk (Alférez et al., 2006). Medium chain fatty acids are absorbed within the intestinal cells without reesterification, directly entering portal circulation and metabolized to

Table 3
Digestive and metabolic utilization of Zn in control and anaemic groups fed with normal-Fe diets either normal or double Ca content

Group	Cow milk diet		Goat milk diet		Level of significance[a]			
	Control	Anaemic	Control	Anaemic	RSD[b]	Diet[c] (D)	Anaemia (A)	Ca supplement
Zn intake ($\mu g\ day^{-1}$)								
Normal Ca	569	571	529	522	62.7	NS	*	NS
Double Ca	583	591	564	561				
Faecal Zn ($\mu g\ day^{-1}$)								
Normal Ca	404a	373A	299b	253B	45.4	***	***	***
Double Ca	373a	367A	253bC	237B				
ADC[d] (%)								
Normal Ca	28.2a	35.8Ac	47.2b	55.9Bc	11.8	***	***	***
Double Ca	32.7a	37.2A	57.3bC	62.2BC				
Urinary Zn ($\mu g\ day^{-1}$)								
Normal Ca	8.4	9.7	7.3	13.9	2.7	***	***	***
Double Ca	11.1	10.1	10.3	8.5				
Zn balance ($\mu g\ day^{-1}$)								
Normal Ca	156.2a	208.8Ac	222.5b	255.3Bc	36.5	***	***	***
Double Ca	199.0aC	233.1Ac	300.9bC	313.9BC				
R/I[e] (%)								
Normal Ca	26.7a	34.2Ac	45.7b	52.9Bc	9.8	***	***	***
Double Ca	32.8aC	35.6A	55.4bC	58.6BC				

[a] Levels of significance are: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; NS, not significant. Interactions: diet × Ca supplement: ADC and *R/I* $P<0.05$; balance $P<0.01$; diet × anaemia × Ca supplement: ADC and *R/I* $P<0.001$. Mean values among groups of controls rats with different lower case letters (a or b) in the same row indicated significant difference by Tukey's test. Mean values among groups of anaemics rats with different upper case letters (A or B) in the same row indicated significant difference by Tukey's test. Mean values that were significantly different from the corresponding group of control rats at $P<0.05$ by Student's *t* test are indicated by alower case letter c. Mean values were significantly different from the corresponding group of rats fed with normal Ca content at $P<0.05$ by Student's *t* test are indicated by an upper case letter C.
[b] RSD, residual standard deviation.
[c] Diet, main effect of diet; Anaemia, main effect of anaemia; Ca supplement, main effect of Ca supplement.
[d] ADC, apparent digestibility coefficient.
[e] *R/I*, balance × intake^{-1}.

obtain energy (Garcia-Unciti, 1996). According to Tappenden et al. (1997), this may favour the transport of nutrients, including Zn, through the basolateral membrane of the enterocyte.

The higher digestive and metabolic utilization of Zn ($P<0.001$) in the anaemic rats than in the controls, regardless of the type of diet consumed, especially in normal Ca content diets (Table 3), may, according to Campos et al. (1998) and to King and Keen (1999), be due to the deficiency of Fe in the intestinal region that produces an increase in the absorption of other divalent cations. However, Gómez-Ayala et al. (1998) demonstrated that in a situation of Fe deficiency, a greater absorption of Cu occurs, while that of Zn remains unchanged.

Supplementation with Ca increased Zn absorption (ADC), balance and *R/I* in control and anaemic rats fed goat milk diet ($P<0.001$), but only in control rats fed cow milk diet for balance and *R/I* ($P<0.01$) (Table 3). The effect of excess Ca on Zn absorption remains controversial: our results are in disagreement with those reported in other studies that showed that an excess Ca diet either did not affect (Takasugi et al., 2007) or decreased (Dursun & Aydogan, 1994; Wood & Zheng, 1997) the absorption of Zn. However, in our study, the supplementation of Ca in the diet increased the nutritive utilization of Zn in both in the control and the anaemic rats for the goat milk diet and in the control rats for the cow milk diet. In contrast to previous reports, in the current study the Ca is supplied in part by the milk (cow or goat) and this can be a key factor in the improvement of the Zn nutritive utilization, especially with the goat milk diet, because of its habitual consumption increases the mineral bioavailability, even in NFA (Alférez et al., 2006; Campos et al., 2007; Nestares et al., 2008).

The interactions of diet × anaemia and anaemia × supplement were not significant but the interaction diet × Ca supplement for ADC, balance and *R/I* were significant at $P<0.05$, 0.01 and 0.05, respectively (Table 3).

The interaction of diet × anaemia × supplement was significant at $P<0.001$ for ADC and *R/I* (Table 3). These interactions reveal that the effect of the Ca supplement was more important when the goat milk diet was consumed by the anaemic rats compared with the cow milk diet.

3.2. Zn concentrations in organs

In general, Zn content in organs was higher for the animals fed with goat milk diet ($P<0.001$) than for those given the cow milk diet (Table 4). The main reason why the goat milk diet (normal and double Ca content) favours Zn deposit in the organs (femur, testes, sternum, spleen, liver and brain), compared with the cow milk diet, is that dietary goat milk increases Zn absorption and retention (by about 60%, Table 3) compared with the cow milk diet, a fact that may explain the higher Zn deposition in the organs studied.

In general, in all the organs studied especially the testes ($P<0.001$) and sternum ($P<0.01$) Zn concentrations were higher in anaemic rats than among their controls for the two milk-based diet assayed, for both normal and double Ca content (Table 4). This fact is in agreement with the greater digestive utilization and *R/I* of Zn in the anaemic rats than in the controls. Zn deposits in the bone (femur) were higher than in the other organs studied. According to Bobilya et al. (1994) osseous deposit seems to provide a utilizable source or reserve of Zn that varies according to the consumption of the mineral. In our study, after the femur, the highest Zn content was found in the testes, this finding is in accordance with King and Keen (1999). In descending order of Zn content, these were followed by the sternum, kidney, spleen, liver, heart and brain. In all of

Table 4
Zn concentrations[a] in several organs in control and anaemic groups fed with normal-Fe diets either normal or double Ca content

Group	Cow milk diet		Goat milk diet		Level of significance[b]			
	Control	Anaemic	Control	Anaemic	RSD[c]	Diet[d] (D)	Anaemia (A)	Ca supplement
Femur								
Normal Ca	227a	231A	306b	326B	47.0	***	NS	***
Double Ca	211aC	221A	285bC	300BC				
Testes								
Normal Ca	197a	235c	218b	245c	33.4	***	***	*
Double Ca	222C	240	242C	239				
Sternum								
Normal Ca	164a	187Ac	177b	199Bc	22.9	***	*	***
Double Ca	159a	164AC	176b	204Bc				
Kidney								
Normal Ca	134	141	145	153	25.2	**	NS	NS
Double Ca	132	153	150	165				
Spleen								
Normal Ca	105a	94A	133b	129B	6.3	***	NS	NS
Double Ca	115	110	118	112				
Liver								
Normal Ca	117	118	118	122	9.8	***	NS	***
Double Ca	117	123A	129C	137BC				
Heart								
Normal Ca	90.4	91.7	96.4	98.5	11.1	***	NS	NS
Double Ca	92.5	94.2	101.8	103.5				
Brain								
Normal Ca	69.6a	79.5A	84.3b	91.1B	12.2	***	NS	NS
Double Ca	76.6	78.1	78.4	86.1				

[a] All Zn concentrations are given as $\mu g\, g^{-1}$ dry weight.
[b] Levels of significance are: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant. Interaction: diet × Ca supplement: femur $P < 0.001$. Mean values among groups of controls rats with different lower case letters (a and b) in the same row indicated significant difference by Tukey's test. Mean values among groups of anaemics rats with different upper case letters (A and B) in the same row indicated significant difference by Tukey's test. Mean values that were significantly different from the corresponding group of control rats at $P < 0.05$ by Student's t test are indicated with a lower case letter c. Mean values that were significantly different from the corresponding group of rats fed with normal Ca content at $P < 0.05$ by Student's t test are indicated with an upper case letter C.
[c] RSD, residual stardard deviation.
[d] Diet, main effect of diet; Anaemia, main effect of anaemia; Ca supplement, main effect of Ca supplement.

these, exchange was found to be rapid, while it was slow in the central nervous system and in bone, which are subject to hormonal regulation (Dunn & Cousins, 1989; Henkin et al., 1984). According to Miller et al. (1994), the exchangeable reserve of Zn is very small, and so a deficiency occurs rapidly when there is a failure of adaptation to consumption (Golden, 1989).

The Ca supplement increased the Zn deposit in liver of control and anaemic rats fed the goat milk diet ($P < 0.05$) and in testes of control rats fed cow and goat milk diets ($P < 0.01$) (Table 4). This is in agreement with Shackelford et al. (1994) who showed that moderate increases in dietary calcium lead to a dose-related linear increase in Zn liver content.

4. Conclusion

Goat milk, especially when Ca supplemented, improved Zn metabolism increasing its digestive and metabolic utilization and its deposit in target organs in control rats and mainly in those with NFA. Inclusion of goat milk in the diet of people who need dietary Ca supplements in a situation of NFA could have positive health implications.

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