Effects of dietary calcium on the metabolism of trace elements in male and female rats

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The effect of dietary calcium on the metabolism of iron, zinc, copper, and manganese in male and female rats was investigated. For 3 or 6 weeks the rats were fed three diets containing: (1) 0.26, (2) 0.52, or (3) 2.08% Ca. The apparent absorption of iron was depressed by the high calcium diet, and manganese absorption was highest in the low calcium groups. Generally there was a decrease in the absorption of minerals from 3 to 6 weeks. With an increase in the dietary calcium the absorption of Ca and P decreased. The liver iron concentration in the females fed diet 3 decreased from about 600 to 200 μg/g dry weight. The high calcium intake also caused a slight increase in the heart calcium levels in both sexes. However, diet 3 prevented kidney calcification in the female rats at 6 weeks and this was attributed to a dramatic decrease in the urinary phosphorus, although the calcium had increased about 40 times. In males, on the other hand, the high calcium diet caused some kidney calcification.

Keywords: calcium, trace elements, interaction, rats

Introduction

In recent years there has been a phenomenal increase in the promotion and sale of calcium supplements.[1,2] The consumer is not sure, however, about the long-term safety of calcium supplementation. There are concerns regarding an increased risk of kidney calcium stones and the interference with the absorption of other minerals.[2] Adverse effects of high calcium intake on the absorption and/or the retention of iron in postmenopausal women[3] and in rats[4,5] have been reported. Mahoney et al.,[5] reported that the iron absorption and the liver iron concentration in male rats decreased as dietary calcium (as $CaCO_3$) increased, even in the presence of lyophilized meat.[5] It was suggested that there may be an increase in the incidence of milk-alkali syndrome including nephrocalcinosis and the impairment of kidney function, due to the popularity of calcium carbonate supplements for the prevention or treatment of osteoporosis.[6] Under normal conditions, such an effect of a high calcium intake has not been observed in man, but milk-alkali syndrome was reported in patients treated with a daily dose of 4 to 10 g calcium as calcium carbonate.[6] The evidence regarding nephrocalcinosis in female rats has shown that changing the calcium/phosphorus weight ratio in the diet from about 0.7 to 1.2, by increasing the calcium concentration, reduced the incidence of kidney calcification virtually to nil.[7] From these reports, it was clear that the effects of dietary calcium on the metabolism of trace elements such as iron, zinc, copper, or manganese and their tissue levels in both sexes, needed to be studied. Moreover, it was necessary to investigate the calcification of soft tissues, like heart and kidney, in relation to dietary calcium. The present rat study was therefore undertaken to investigate the effect of dietary calcium on the metabolism of iron, zinc, copper, and manganese and on the calcification of heart and kidney.

Materials and methods

Diets

The diets contained (in percent): vitamin-free casein, 24.5 providing 20% protein; sucrose, 20.0; cellulose,

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6.0; corn oil, 10.0, AIN-76A[8] vitamin mix, 1.0; AIN-76[8] mineral mix without calcium phosphate which was replaced by sucrose, 3.5; DL-methionine, 0.3; choline bitartrate, 0.2; corn starch, to make 100. Calcium phosphate (mono basic) and oxide were added to give 0.4% P in all the three diets and 0.26% (Diet 1), 0.52% (Diet 2) and 2.08% Ca (diet 3). Thus the Ca:P weight ratios were 0.65, 1.3 and 5.2, respectively.

Animals

Six weeks old Sprague-Dawley rats, 35 of each sex, were purchased from Charles River Canada Inc., St. Constant, Québec. Ten males per group and an equal number of females were randomly allocated to three diets. They were housed in wire-bottomed stainless-steel cages and food and distilled water were available by free choice. Illumination was provided automatically from 7 AM to 7 PM. Room temperature was controlled at 22 ± 1°C and humidity at 45–55%. Body weights were recorded weekly.

Methods

At the beginning, 5 males and 5 females were given an intraperitoneal dose of pentobarbital (30 mg/kg body weight) and sacrificed by exsanguination from the abdominal aorta. Heart, liver, femur, and kidneys were removed and kept frozen at −20°C until analyzed for the different minerals by flame atomic absorption spectroscopy[9] and phosphorus by colorimetry.[10]

During the second and the fifth week of feeding, the rats were trained, for 3 days, to eat their daily diet between 8:30 AM and 1:00 PM and then placed in plastic (Nalgene) metabolic cages from 1:00 PM to 8:30 AM the following day for the collection of urine and feces. In the metabolic cages, distilled water was available to the animals but food was not, so that urine would not be contaminated with food. Urine and feces were collected for 3 days. The urine, acidified with 1 ml of 6 N HCl and feces were stored at −20°C until analyzed as described above.

At the end of weeks 3 and 6, 5 males and 5 females were sacrificed as described above. The tissues were removed and stored at −20°C until analyzed for the different minerals.

The diets were analyzed for the minerals by atomic absorption spectroscopy and colorimetry. Calcium, phosphorus (0.57%), and magnesium (0.06%) were higher than expected because of the contribution from sources in the diet other than the salt mixture.

Statistics

In the examination of the data three factors (sex, time, and diet) were considered. Analysis of variance procedures[11] were used. Interactions between the factors were tested by pairs using Bonferroni's procedure.[12] F-tests with p-values less than 5% were considered significant. Since the kidney calcium data were not normally distributed, nonparametric procedures were used. The data were ranked ignoring sex and the level of dietary calcium and the ranks were analyzed by multivariate analysis of variance.[13] Significant analyses were followed by univariate analysis of variance for each variable.[14]

Results and discussion

The body weights at 3 and 6 weeks, given in Table 1, show that the high calcium diet had an adverse effect on the growth of both male and female rats. At 6 weeks, males fed the high calcium diet weighed about half as much as those which received 0.52% Ca—the AIN recommended level.[8] The corresponding difference in females was somewhat less. From the food intakes measured during the two metabolic periods, it was evident that the high calcium diet caused hypophagia, which was responsible for the depressed growth. Male weanling rats fed the AIN-76 diet, containing 0.8 or 3.0% calcium, for 4 weeks, however, were reported to consume equal amounts of the diets.[15] Similarly, diets containing 0.17 to 2.35% calcium fed to young dairy calves did not show any effect on food intake or weight gain, the NRC recommended level for calves being 0.4%.[16,17]

The liver weights/100 g body weight (Table 2), also indicated the rats fed the high calcium diet had smaller livers than the rats in the other two diet groups. At 6 weeks the relative liver weights of the males and females were 65 and 80% of the corresponding values for the animals that were fed the 0.52% Ca diet.

The data on the apparent absorption of calcium, phosphorus, and magnesium are presented in Table 3. The sex of the animal did not have any significant effect on the absorption of these minerals. As the dietary calcium increased, the absorption of both calcium and phosphorus decreased to half or less, but the effect on the magnesium absorption was not consistent. The only significant effect was a decrease in the absorption from the 0.52% Ca diet by both sexes as compared with the other two diets. The absorption of the three minerals at 6 weeks was less than that at 3 weeks. The decrease in the calcium absorption with higher intake, observed here, was not seen by Johnson and Kies[15] in young rats fed diets containing 0.8 or 3% calcium, nor did they report any effect of age from the first to the fourth week of feeding. In adult female rats, calcium absorption was found to decrease when the intake was high.[18] In human subjects, however, the inverse relationship between intake or age and calcium absorption was similar to our observation.[19,20] The amount of calcium absorbed from the high calcium diet was high but the urinary excretion was very high (Table 9) probably resulting in retention which was not much higher than from the diet containing 0.52% Ca.

The decrease in the phosphorus absorption due to the high calcium intake was in agreement with the results reported in adult female Sprague-Dawley rats.[18] The reports on the effect of high calcium on magnesium absorption in animals and man have not been consistent.[21] Our results do not support the concept of

Table 1 Body weights (grams)

Dietary Ca, (percent)	Week	Male		Female	
		Initial	Final	Initial	Final
0.26	3	139 (7)[a]	321 (4)	127 (2)	215 (2)
	6	143 (3)	379 (15)	125 (4)	247 (19)
0.52	3	146 (4)	312 (11)	124 (4)	222 (7)
	6	143 (5)	426 (23)	126 (3)	273 (32)
2.08[b]	3	148 (6)	176 (13)	131 (4)	145 (21)
	6	143 (16)	220 (36)	127 (3)	203 (20)

[a] Mean (standard deviation), N = 5.
[b] Final weights at 3 and 6 weeks, less than the corresponding weights for the other two diet groups, $P < 0.01$.

Table 2 Liver weight (g/100 g body weight)

	Dietary Ca (percent)					
	0.26		0.52		2.08[b]	
	M	F	M	F	M	F
Initial	3.7(0.1)[a]	3.4(0.5)				
Week 3	4.9(0.6)	4.3(0.3)[c]	4.5(0.2)	4.2(0.2)[c]	3.1(0.2)	3.3(0.3)
Week 6[d]	4.3(0.7)	3.2(0.2)[c]	4.2(0.7)	3.6(0.5)[c]	2.7(0.3)	2.9(0.2)

[a] Mean (standard deviation), N = 5.
[b] Less than other groups, $P < 0.001$.
[c] Less than males, $P < 0.05$.
[d] Less than week 3, $P < 0.01$.

Table 3 Apparent absorption of calcium, phosphorus and magnesium (percent)

Dietary Ca (percent)	Diet (No.)	Male		Female	
		Week 3	Week 6	Week 3	Week 6
Calcium[b]					
0.26	1	92(2)[a]	85(6)[e]	94(2)	78(3)
0.52	2	65(10)	50(6)	84(9)	48(6)
2.08	3	52(14)	46(16)	47(16)	43(14)
Phosphorus[c]					
0.26	1	94(1)	92(3)	95(1)	90(1)
0.52	2	78(7)	69(4)	90(5)	69(4)
2.08	3	45(13)	41(16)	40(15)	37(15)
Magnesium[d]					
0.26	1	82(4)	77(7)	84(4)	76(4)
0.52	2	70(8)	59(5)	86(9)	58(7)
2.08	3	80(7)	75(9)	74(11)	76(11)

[a] Mean (standard deviation), N = 5.
[b] Week 6 less than week 3, $P < 0.01$; diet 1 higher than diet 2 and 3 and diet 2 higher than diet 3, $P < 0.01$.
[c] Week 6 less than week 3, $P < 0.01$; diet 1 higher than diet 2 and diet 2 higher than diet 3, $P < 0.01$.
[d] Week 6 less than week 3, $P < 0.01$; diet 2 less than diet 1 and 3, $P < 0.05$.

competitive inhibition of magnesium absorption by a high calcium intake.

The results given in Table 4 indicate that there were wide variations in the absorption of the trace elements. Consequently, the interpretation of these data has to be cautious. The diet effect was significant in the case of iron and manganese. High calcium intake depressed iron absorption but manganese absorption was lowest in the low calcium groups. The decrease in absorption due to age was significant except for manganese. The absorption of iron, zinc, and copper was not affected by the sex of the rats, but the manganese absorption by the females was somewhat less than that by the males. The adverse effect of high calcium on the absorption of iron but not of zinc was similar to that reported in women, who were given a test meal having a Ca:P (weight) ratio of 2.8.[3] The inhibition of iron absorption by high dietary Ca was in

Table 4 Apparent absorption of iron, zinc, copper, and manganese (percent)

Dietary Ca (percent)	Diet (No.)	Male		Female	
		Week 3	Week 6	Week 3	Week 6
Iron[b]					
0.26	1	58(12)[a]	49(12)	45(10)	41(9)
0.52	2	67(13)	36(4)	64(15)	33(13)
2.08	3	45(12)	36(18)	36(15)	35(14)
Zinc[c]					
0.26	1	49(9)	45(10)	31(12)	32(10)
0.52	2	45(9)	32(9)	56(18)	22(10)
2.08	3	29(16)	27(18)	30(11)	29(15)
Copper[d]					
0.26	1	35(13)	27(12)	16(18)	17(17)
0.52	2	34(9)	14(13)	45(23)	11(16)
2.08	3	21(15)	17(22)	22(12)	15(21)
Manganese[e]					
0.26	1	20(14)	25(10)	−4(21)	11(20)
0.52	2	51(20)	20(9)	37(26)	12(18)
2.08	3	36(15)	29(23)	28(9)	28(18)

[a] Mean (standard deviation), N = 5.
[b] Week 6 less than week 3, $P < 0.01$; diet 3 less than diet 1 and 2, $P < 0.05$.
[c] Week 6 less than week 3, $P < 0.05$.
[d] Week 6 less than week 3, $P < 0.01$.
[e] Diet 1 less than diet 2 and 3, $P < 0.05$; male higher than female, $P < 0.05$.

agreement with that reported by Barton et al.,[4] who used a diet having Ca:P = 3.8. Earlier studies had indicated that phosphate alone, unless at very high levels, and calcium did not block the absorption of non-heme iron in man.[27] More recent reports have shown, however, that the inhibition of iron absorption by calcium as calcium chloride or by milk containing the same amount of calcium and a higher level of phosphorus was the same.[28] Thus the presence of high phosphate is not essential for the inhibition of iron absorption by high dietary calcium. It would be interesting, however, to determine the effect of increasing the phosphate level on the metabolism of trace elements. The Ca:P ratio in the case of women consuming a supplement of 2,000 mg of calcium would be over 2.5.[2] As mentioned above, the inhibition of the absorption of iron by women was observed when the Ca:P ratio in the test meal was 2.8.[3] Just as this ratio did not have any adverse effect on zinc absorption, we did not observe any effect of a much higher Ca:P ratio (5.2).

The liver levels of these trace elements, which should be considered with the absorption data, are summarized in Tables 5 and 6. Before receiving the purified diets, the rats were maintained on a stock diet (Purina 5001) which, according to the manufacturer, contained higher levels (μg/g) of iron (200 vs. 58), zinc (70 vs. 42), copper (18 vs. 6), and manganese (64 vs. 54) than that provided by the AIN-76[8] salt mixture. Consequently the iron, zinc, and copper levels in the livers of the rats generally declined at 3 weeks. The effect of the change from the stock diet to the purified diet on the liver manganese concentration was small and inconsistent, probably because the difference in the manganese content of the diets was relatively small. The adverse effect of the increased dietary calcium on the iron absorption by male rats reported earlier[5] was confirmed here in both sexes. The decrease in liver iron due to the high calcium was seen in the females but not in the males as reported by these workers. This may be attributed to the difference in the source of iron (meat vs. ferrous sulphate) or in the age of the rats (6 vs. 9–12 weeks). Although earlier studies in man had indicated that the presence of both calcium and phosphate was essential for an inhibition of iron absorption,[22] recent results[3] have shown that calcium alone (as $CaCO_3$) had a similar effect. In rats also, calcium alone was found to have an adverse effect on iron absorption.[4,5]

There was no significant effect of the dietary calcium, or sex on the zinc absorption but there was a decrease from 3 to 6 weeks. The liver zinc concentrations in both males and females fed the high calcium diet were about 20% higher than those in rats maintained on the other two diets. No change in the liver zinc was seen, however, from 3 to 6 weeks. An increase of the dietary calcium was reported to reduce the zinc absorption in rats but in human volunteers this effect was not seen.[23]

The liver copper levels in the females were generally higher than those in the males probably because female rats are more efficient in the utilization of dietary copper, as reported earlier.[24,25] The apparent absorption of copper (Table 4), however, did not show any significant difference between the sexes. There was a decrease in the copper absorption from 3 to 6

Table 5 Iron and zinc in liver (μg/g dry weight)

	Dietary Ca (percent)					
	0.26		0.52		2.08	
	M	F	M	F	M	F
Iron[b]						
Initial	391(44)[a]	487(108)				
Week 3	196(21)	542(40)	183(26)	471(87)	195(55)	334(213)[d]
Week 6	237(59)[c]	622(105)[c]	240(36)[c]	616(181)[c]	157(29)[e]	199(23)[d,e]
Zinc						
Initial	105(4)	108(6)				
Week 3	74(3)	77(1)	73(5)	78(5)	92(4)[f]	92(3)[f]
Week 6	73(16)	85(6)	73(3)	80(10)	93(6)[f]	100(14)[f]

[a] Mean (standard deviation), N = 5.
[b] Higher in females than males, $P < 0.01$.
[c] Higher than week 3, $P < 0.05$.
[d] Less than females in other diet groups, $P < 0.01$.
[e] Less than week 3, $P < 0.05$.
[f] Higher than other diets, $P < 0.01$.

Table 6 Copper and manganese in liver (μg/g dry weight)

	Dietary Ca (percent)					
	0.26		0.52		2.08	
	M	F	M	F	M	F
Copper						
Initial	13.8(0.6)[a]	14.6(1.7)				
Week 3	11.7(0.8)	13.2(0.6)[b]	11.8(0.5)	13.5(0.6)[b]	13.5(0.6)[c]	13.1(1.1)
Week 6	11.9(2.0)	15.6(2.4)[b,d]	11.0(0.7)	13.3(1.1)[b]	13.9(1.3)[c]	16.1(1.8)[b,c]
Manganese						
Initial	8.5(0.9)	8.5(0.8)				
Week 3	8.8(0.6)	8.5(0.4)	8.1(0.9)	8.0(0.3)	9.2(0.9)	7.8(1.2)
Week 6	6.9(1.2)[e]	8.4(0.6)[b]	6.7(0.2)[e]	7.5(1.0)	9.0(0.8)[f]	9.6(0.9)[f]

[a] Mean (standard deviation), N = 5.
[b] Higher than males, $P < 0.05$.
[c] Higher than males in other diets, $P < 0.05$.
[d] Higher than week 3, $P < 0.05$.
[e] Less than week 3, $P < 0.05$.
[f] Higher than other diets for the same sex, $P < 0.05$.

weeks in both sexes, but this was not reflected in the liver copper content.

The apparent manganese absorption was lower in the low calcium groups than in the other two diet groups but at 6 weeks the liver levels in the rats fed the low and medium calcium diets were less than in those fed the high calcium diet. A low calcium/phosphorus ratio in rat diets was reported to enhance manganese absorption,[26] but this effect was not observed in our low calcium diet groups, based either on the apparent absorption or the liver concentration.

In regard to soft tissue calcification, the results in Table 7 show a 10–20% increase in the heart calcium levels in both sexes and a similar increase in the heart phosphorus concentration in the female rats at 6 weeks. Among the adverse effects of a high calcium intake, an increased risk of kidney stones and an inhibition of the absorption of other minerals are often mentioned,[1,2] but the increase in the heart calcium observed here was not considered likely. Whether the calcification of the heart would have increased further on prolonged feeding of the high calcium diet needs to be determined.

Since the variation in the kidney calcium levels was large, mean, median, minimum and maximum concentrations are given in Table 8. The number of animals having a calcium concentration above 0.5 mg/g dry weight is also included in the table. From our earlier work on nephrocalcinosis,[27] this calcium level was found to be the upper limit in normal rat kidneys. Surprisingly the females fed the high calcium diet did not show any nephrocalcinosis whereas all the kidneys of females in the other groups were highly calcified. On the other hand, the high calcium diet caused some kidney calcification in the male rats. The kidneys of all the five male rats that received the AIN-76[8] recom-

Table 7 Minerals in heart at 6 weeks (μg/g dry weight)

	Dietary Ca (percent)					
	0.26		0.52		2.08	
	M	F	M	F	M	F
Calcium	158(8)[a]	142(6)	146(8)	151(16)	179(14)[b]	170(4)[b]
Phosphorus	9240(620)	8150(500)	8530(640)	8650(510)	8810(660)	10180(1400)[b]
Magnesium	657(22)	601(31)	616(51)	618(41)	637(56)	669(42)

[a] Mean (standard deviation), N = 5.
[b] Higher than other diets for the same sex, $P < 0.01$.

Table 8 Kidney calcium at 6 weeks (mg/g dry weight)

	Dietary Ca (percent)					
	0.26		0.52		2.08	
	M	F	M	F	M	F
Mean	0.62	16.05[b]	0.30	11.65[b]	0.88	0.35[a]
Median	0.30	13.08	0.28	10.37	0.77	0.34
Minimum	0.23	0.55	0.27	3.36	0.31	0.32
Maximum	2.00	41.89	0.37	22.70	1.66	0.40
No. > 0.50	1/5	5/5	0/5	5/5	2/4	0/5
No. in group						

[a] Less than females in other diet groups, $P < 0.01$.
[b] Higher than males, $P < 0.01$.

Table 9 Week 6 urinary mineral excretion (mg/g creatinine)

	Dietary Ca (percent)					
	0.26		0.52		2.08	
	M	F	M	F	M	F
Calcium	19(4)[a]	39(24)[c]	19(6)	40(21)[c]	2154(837)[b]	1555(413)[b]
Phosphorus	3255(283)	3865(168)	2768(472)	2760(415)	2.2(0.4)[d]	5.2(3.6)[d]
Magnesium	273(46)	331(22)	282(58)	266(42)	405(61)[e]	375(52)

[a] Mean (standard deviation), N = 5.
[b] Higher than other diets, $P < 0.01$.
[c] Higher than males, $P < 0.05$.
[d] Less than other diets, $P < 0.01$.
[e] Higher than other diets, $P < 0.05$.

mended level (0.52% Ca) were not calcified, but in a larger group of 12 rats, some rats did have increased calcium levels.[28] This is due to the fact that nephrocalcinosis is a discrete rather than homogeneous phenomenon.[7] The prevention of kidney calcification in the female rats receiving the diet having a high Ca:P weight ratio of 4 confirms the earlier observation of Clapp et al. in Wistar rats.[7] Whether a dietary Ca:P weight ratio of less than 4 can prevent nephrocalcinosis in both sexes remains to be ascertained.

From the urinary excretion data (Table 9), it is clear that the largest changes occurred in the calcium and phosphorus. While the calcium excretion by rats in the high calcium group increased about 40 to 100 fold that in animals fed 0.52% Ca, the urinary phosphorus went down from 2,700 to 2–5 mg/g creatinine. The prevention of the precipitation of calcium phosphate can be attributed to the lack of sufficient phosphate in the filtered fluid. A similar effect of a decreased urinary phosphorus was observed by us earlier.[27] It is interesting that even when the females were fed a low calcium (0.26%) diet, the urinary calcium excretion was almost twice that in male rats. The urine of both the males and females fed the diet containing twice the level of calcium had comparable calcium levels in this and an earlier experiment.[27]

In summary, the high calcium diet inhibited the growth of male and female rats and also had some adverse effects on the liver weight/100 g body weight. The apparent absorption of calcium and phosphorus decreased from 3 to 6 weeks. It was also reduced as the dietary calcium increased. The iron, zinc and copper absorption also decreased from 3 to 6 weeks but the high dietary calcium depressed the iron absorption

and the absorption of manganese was lowest in the low Ca groups. The apparent absorption of manganese was not significantly affected by age. There were no consistent differences between the absorption of the minerals by the males and females. The liver iron concentration decreased from 3 to 6 weeks in the animals fed the high calcium diet, but increased in the rats fed the other diets. The high calcium diet diminished the liver iron level in the females from about 600 to 200 μg/g dry weight. There were some minor effects on the liver concentrations of the other minerals.

The heart calcium content increased slightly in the animals fed the high calcium diet. Kidney calcification was prevented by this diet in female rats and this was attributed to the dramatic decrease in the urinary phosphorus, although the calcium had increased about 40 fold. The high calcium diet, however, caused some nephrocalcinosis in male rats.

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