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Dietary Calcium Had No Reducing Effect on Body Fat and Weight Gain in Sprague-dawley Rats

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Abstract: Recently, studies have focused on the effects of dietary calcium on the weight and fatness. Some of these studies have indicated that there is negative relationship between dietary calcium and body fat, while others have reported no such effects. There are also controversies over serum parathyroid hormone, as its mechanism, on body fat and weight. The objective of the present study was to evaluate the effects of three dietary calcium levels (0.2%, 0.5% or 1.2% Ca^{2+}) on body fat and weight gain in male Sprague-Dawley rats, using same diet composition for nutrients other than calcium. The study duration was 72 days. At the end, truncal blood samples were drawn from decapitated rats to measure the effects of diets on serum calcium, PTH and vitamin D. The carcasses were minced and homogenized to measure their body fat percent by the methods of Soxhelet. There were no significant effects of dietary calcium on food intake ($p>0.05$), body weight gain ($p>0.05$) or carcass fat content ($p>0.05$), while the serum PTH levels were inversely related to calcium intake ($p<0.05$). In conclusion, our findings do not support the effects of dietary calcium and parathyroid hormone on body fat and weight.

Key words: Dietary calcium, body fat, weight, PTH

Introduction

The effects of a variety of macronutrients, particularly carbohydrates and fats, on changes in body fat and weight have been frequently studied. However the role of micronutrients has received less attention (Lin *et al.*, 2000; Loos *et al.*, 2004). Calcium is a micronutrient now receiving much attention as a potential modifier of body composition and weight (Lin *et al.*, 2000; Loos *et al.*, 2004; Shi *et al.*, 2001; Zemel *et al.*, 2000; Sun and Zemel, 2004; Zhang and Tordoff, 2004; Paradis and Cabanac, 2005; Sakhaee and Malouf, 2005; Boon *et al.*, 2005; Lanou, 2005; Teegarden, 2005; Papakonstantinou *et al.*, 2003). There are great controversies over the relationship between calcium, body composition and weight in both human and animals. Few recent studies on animals suggested an anti obesity effect for calcium (Shi *et al.*, 2001; Zemel *et al.*, 2000; Sun and Zemel, 2004; Papakonstantinou *et al.*, 2003), while others have shown no such relationship (Zhang and Tordoff, 2004; Paradis and Cabanac, 2005). Human studies are also controversial and complicated by various confounders (Sakhaee and Malouf, 2005; Boon *et al.*, 2005; Lanou, 2005). In some studies the calcium source has been based on milk and therefore it may overlap by the bioactive compounds in milk and dairy products (Zhang and Tordoff, 2004; Paradis and Cabanac, 2005; Teegarden, 2005).

The present study was designed to assess the effects of dietary calcium on body fat and weight, while controlling for other food ingredients in the absence of milk as a source of dietary calcium.

Materials and Methods

Animals and diets: Forty eight male, 10 weeks old Sprague-Dawley rats from Razi Institute of Iran were used. The rats were kept on regular diet for adaptation for a week and then were divided randomly in three groups and fed ad libitum on a low calcium (LC: 0.2 percent W/W), regular calcium (RC: 0.5 percent W/W) or high calcium diet (HC: 1.2 percent W/W) based on purified AIN-93M diet (Table 1). The vitamin mix and mineral mix, except for $CaCO_3$, were based on AIN-93M-MX (Reeves *et al.*, 1993). The $CaCO_3$ content of mineral mix, in LC group was 140 grams and the remainder was filled with finely powdered sucrose. The $CaCO_3$ contents of LC, RC and HC diets were 0.49, 1.25 and 3.0 grams percent anhydrous calcium carbonate (40.4% Ca), respectively.

Rats were kept individually in stainless steel cages within a room with 22-25 degree centigrade temperature, 50-60% humidity and 12 h dark-light cycle (6AM-6PM). They were fed ad libitum and had free access to de-ionized water. All animals survived up to the end of study except one.

Table 1: Ingredients of diets used for feeding the rats in three groups

Ingredient	Amount LC	RC	HC
Cornstarch	465.692	465.692	465.692
Casein(= 85% Protein)	140	140	140
Dextrinized corn starch	155	155	155
Sucrose	100	100	100
Soy bean oil	40	40	40
Fiber(alpha cellulose)	50	50	50
Mineral Mix	35	35	35
Vitamin Mix	10	10	10
L-cysteine	1.8	1.8	1.8
Choline bitartrate (41.1%)	2.5	2.5	2.5
TBHQ	0.008	0.008	0.008
Additional Calcium Carbonate	-	-	12.5

Measurements: The study duration was 72 days and the rats were weighed in 9 day's period. Food intakes of rats were measured in three-day periods to the nearest 0.5 of gram. Spillages were measured and subtracted from the eaten foods. From the second week of the study rat drops were collected and fecal fat percent was measured by the method introduced by Papakonstantinou *et al.* (2003). Five grams of rat droppings dried in the room temperature were separated. Feces were grounded and saponified by potassium hydroxide in ethanol containing 0.4% isoamyl alcohol at 80°C and then the fatty acids were liberated by adding HCl to the alkaline solution. The fatty acids were then extracted with petroleum ether at 40°C for an hour. The extraction was pooled in a weighed container and dried. Lipid content was determined by weighing the extraction. The amount of fecal lipid was expressed as a percentage of the weight of the starting fecal sample. At the end of the study rats were anaesthetized with ether and then decapitated. The truncal blood was drained in three 1.5 mL microtubes. Blood samples were kept in laboratory medium for half an hour and then centrifuged at 2000 g for 15 min. Serum samples were drained in 500 λ microtubes and stored at-80 degree centigrade.

Ionized calcium concentrations of serum samples were determined by ionized calcium analyzer (Easylyte Na/K/Ca/ PH, Medica Corporation).

Total serum calcium was measured by cresolphthalein complexone. Serum parathyroid hormone was measured using radio immunoassay method. 25 hydroxy Cholecalciferol (Calcidiol) was measured by radioimmunoassay method. Weight changes are computed by subtracting baseline weight from final weight.

Carcass composition: The carcasses were shaved; their stomachs were emptied and they were frozen at-20 degree centigrade without head and tail. Thirty days later carcasses were sliced in small pieces and minced twice by meat grinder. The minced materials were homogenized by Delonghi homogenizer model KR400P. 100 mL distilled water was added to homogenizing corpus to be easily homogenized. This method is similar to that used by Brooks *et al.* (1995). Carcass lipid content was determined on 3 grams of dried samples of material, using BUCHI automated instrument by Soxhelet's methods (Brooks *et al.*, 1998). Ash content was measured on 5 grams of dried sample using a hot plate and 550 degree centigrade furnace based on method used by Papakonstantinou *et al.* (2003). Carcass protein percent was computed as 100 minus ash and fat percent (Zhang and Tordoff, 2004) based on dry basis.

Statistical analysis: Statistical analyses were performed using the SPSS version 11.5. The data are expressed as mean±standard deviation and confident interval of 95 percent. The groups' means were compared by One-Way ANOVA for single measurements. Repeated Measure ANOVA was used for comparing repeated measures of weight. The mean of serum parathyroid hormone concentrations were compared by Tamhane post hoc test as the homogeneity of variances was not met by levene statistic. The mean concentrations of calcium in three groups were compared by scheffe post hoc test.

Results

Baseline weights of the three groups were similar (Table 2). During the study, the HC group gained more weight than the other two groups. However, the weight differences among the three groups were not statistically significant [$F (2, 44) = 2.57$, $p = 0.088$]. Food intake per gram basal body weight and total food intake during the study did not differ significantly among the 3 groups [$F (2, 44) = 0.26$, $p = 0.77$].

While serum ionized Ca levels were not significantly different among the 3 groups [$F (2, 40) = 1.7$, $p = 0.2$], serum total Ca levels were significantly higher in HC group when compared with the LC group [$F (2, 42) = 3.98$, $P = 0.026$]. On the contrary, serum PTH levels of HC group were significantly lower than the other two groups [$F (2, 38) = 10$, $P = 0.0003$). Serum PTH levels of RC group were also significantly lower than LC group (Tamhane statistics, $p<0.008$). Serum levels of 25-hydroxy Cholecalciferol were not significantly different among the 3 groups [F9 (2, 41) = 2.29, $p = 0.11$]. No significant differences were seen among the 3 groups for body fat [$F (2,44) = 0.55$, $p = 0.58$] and protein contents [$F (2, 44) = 0.67$, $p = 0.52$] and ashes [$F (2,44) = 1.3$, $p = 0.29$] and fecal fat content [$F (2,42) = 0.73$, $p = 0.49$] (Table 2).

Fig. 1 shows weekly weight gain of the three groups during the study. During the first week, the growth velocity of the three groups was fast and slowed down thereafter. The weight gain pattern was similar in the three groups. Although their weight gain was

Table 2: Body weight, food intake, serum parameters and body composition and fecal fat of male Sprague-Dawley rats fed different calcium iet for 10 weeks

Variable	Low Calcium Mean±SD (n)	Regular Calcium Mean±SD (n)	High Calcium Mean±SD (n)
Weight, g			
Baseline	222.9±27.8 (16)	221.6±33.0 (15)	222.6±27.3 (16)
Final	288.2±36.1 (16)	294.2±41.5 (15)	299.6±38.8 (16)
Change	65.31±14.36(16)	71.27±20.59(15)	79.90±19(16)
Food Intake, g			
Per gram baseline body weight	5.97±0.43 (16)	5.94±0.63 (15)	5.83±0.66 (16)
Total	1120±113 (16)	1118±134 (15)	1087±151 (16)
Serum calcium, mg/dl			
Ionized	1.15±0.14 (15)	1.26±0.15 (14)	1.21±0.20 (14)
Total	9.32±0.73 (16)	9.80±0.63 (14)	9.96±0.64 (15)[a]
Serum PTH, pg/ml	42.2±28.5 (13)	23.6±8.7 (14)[d]	12.4±8.8 (14)[b,c]
Serum Calcidiol, pg/ml	10.6±5.4 (16)	9.9±4.9 (14)	7.2±2.3 (14)
Body composition(dry basis)			
Fat%	23.0±6.0 (16)	22.0±5.3 (15)	24.2±5.9 (16)
Protein%	68.4±6.0 (16)	69.7±5.2 (15)	67.2±6.1 (16)
Ash%	8.7±0.7 (16)	8.3±0.7 (15)	8.6±0.6 (16)
Fecal fat	0.76±0.22 (15)	0.76±0.26 (14)	0.85±0.25 (16)

Significant difference between: HC and LC a) p<0.05, b) p<0.001; HC and RC c) p<0.001; LC and RC d) p<0.05

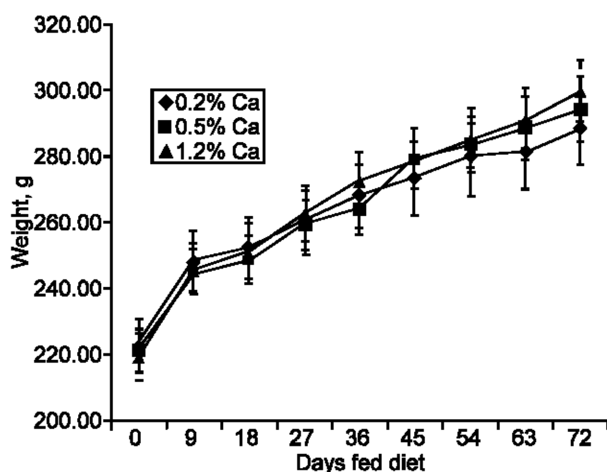

Fig 1: Mean body weight gain of male Sprague-Dawley rats fed isocaloric diets with different levels of calcium

significantly different in term of time [F (2.73, 120) = 330, p<0.0006], their statistic difference was insignificant in term of Calcium levels [F (2, 44) = 0.047, p = 0.95]

Discussion

The objective of this study was to test the hypothesis that dietary calcium might reduce weight gain and body fat. However, we didn't find any significant relationship between weight gain, body fat and the three levels of dietary calcium intake. After 10 weeks, the mean body weight of the rats on the low calcium diet, not only were not higher but also were slightly lower than high-calcium group (p = 0.088). Also the similar fat contents of the three groups of rats do not support the hypothesis of calcium deficiency obesity.

Recent studies on animals to examine the effects of dietary calcium on body fat and weight aroused great controversies. Zhang and Tordoff in a study showed that rats fed the 1.8% Ca^{2+} diets, gained less weight than those fed the 0.2% or 0.6% Ca^{2+} diets (p = 0.0009). But they showed that calcium content of food had no significant effects on fat contents of body (Zhang and Tordoff, 2004). Paradis and Cabanac arranged an experiment to determine the effects of calcium deprivation on body weight set point and suggested no effects of calcium on the set point of body weight and they also found that calcium deprived rats had non significant lower mean body weight (Paradis and Cabanac, 2005).

In contrast to our result, some investigators have reported that a high-calcium diet significantly reduces adipose tissue mass of rats (Papakonstantinou *et al.*, 2003) and mice (Shi *et al.*, 2001; Zemel *et al.*, 2000; Sun and Zemel, 2004). Papakonstantinou *et al.* (2003), using non-fat dry milk as a source of dietary calcium, concluded that the rats receiving high calcium dairy-based diet gained less weight and also had less fat content compared to lower calcium diets. Shi *et al.* (2001) and Zemel *et al.* (2000), in their study on ap2-agouti transgenic mice found that mice on low calcium diet gained more weight and more fat pads than mice on high-calcium diet. Sun and Zemel (2004), in a study to investigate the effects of dietary calcium content and sources in regulating lipid metabolism in response to energy repletion after energy deprivation, showed that both high dietary calcium of dairy sources and $CaCO_3$ supplement reduced fat regain in aP2-gene transgenic mice. However the effect of dairy calcium was higher than $CaCO_3$ supplemented diet.

There are obvious differences between studies supporting dietary calcium effects (Shi *et al.*, 2001;

Zemel *et al.*, 2000; Sun and Zemel, 2004; Papakonstantinou *et al.*, 2003) and those that did not show any significant effects (Zhang and Tordoff, 2004; Paradis and Cabanac, 2005).

The animals in the study of Shi *et al.* (2001), Zemel *et al.* (2000) and (Sun and Zemel, 2004) were ap2-agouti transgenic mice. This type of mice has ectopic agouti expression in comparison with normal mice (Shi *et al.*, 2001; Zemel *et al.*, 2000; Sun and Zemel, 2004; Arora and Anubhuti, 2006) that confers a pleiotropic syndrome characterized by obesity, hyperinsulinemia, peripheral insulin resistance, impaired glucose tolerance, hyperglycemia in males, increased susceptibility to cancer and yellow hair (Michaud *et al.*, 1997; Nelson *et al.*, 1998; Manne *et al.*, 1995). Also they have a genetic lesion influencing calcium metabolism (Zhang and Tordoff, 2004) that makes them susceptible to show extreme variations in response to dietary calcium, as it is shown on the studies (Shi *et al.*, 2001; Zemel *et al.*, 2000; Sun and Zemel, 2004; Stütz *et al.*, 2005).

Incubation of freshly isolated and cultured skeletal muscle myocytes from wild-type mice in agouti-conditioned medium resulted in elevated $[Ca^{2+}]_i$ levels by influencing Ca^{2+}influx but not Ca^{2+}efflux or release from $[Ca^{2+}]_i$ stores (Manne *et al.*, 1995). This suggests that, the primary function of the agouti protein may be directly target to specific subtypes of Ca^{2+}channels. However in normal animals, elaborate mechanisms have evolved to maintain $[Ca^{2+}]_i$ (Manne *et al.*, 1995; Jensen *et al.*, 2004) that is 4 orders of magnitude lower than those found outside the cell (Manne *et al.*, 1995; Anderson *et al.*, 2005).

In addition data have shown that elevating intracellular calcium by depolarization with KCl inhibits lipolysis (Xue *et al.*, 2001), even though adrenergic stimulation elevates intracellular calcium (Seydoux *et al.*, 1996; Boschmann *et al.*, 2002) has shown that it enhances the lipolysis (Boschmann *et al.*, 2002). Thus, the intracellular calcium action on lipolysis is not completely understood, especially with physiological stimuli.

Dairy products could also modulate adipocyte metabolism and obesity risk (Shi *et al.*, 2001; Zemel *et al.*, 2000; Sun and Zemel, 2004; Teegarden, 2005; Teegarden and Zemel, 2003). In studies of Shi *et al.* (2001), Zemel *et al.* (2000) and Papakonstantinou *et al.* (2003), dietary calcium source was both from nonfat dry milk and $CaCO_3$, whereas Zhang and Tordoff (2004), (Paradis and Cabanac, 2005) and we, did not used any milk in our study diets. Notably, dairy products exert significantly greater effects on adiposity than calcium alone (Zemel *et al.*, 2000; Sun and Zemel, 2004). There may be bioactive components in dairy products such as peptides (Teegarden and Zemel, 2003; Dunshea, 2005), branched chain amino acids (Teegarden, 2005; Teegarden and Zemel, 2003; Layman and Baum, 2004), glyco-macro peptide (Dunshea, 2005), casomorphins, caseinomacropeptide (Anderson and Moore, 2004) and angiotensin-converting inhibitory peptide (Teegarden and Zemel, 2003) that could affect body fat and weight (Teegarden, 2005; Teegarden and Zemel, 2003; Anderson and Moore, 2004). Another component of dairy products, conjugated linoleic acid, may also affect body weight (Anderson and Moore, 2004). However, the studies in mice (Shi *et al.*, 2001; Zemel *et al.*, 2000) and rats (Papakonstantinou *et al.*, 2003) that showed an enhanced effect of dairy product on changes in body weight, were completed with a nonfat dairy source (nonfat dry milk powder) and thus devoid of conjugated linoleic acid.

Some preliminary evidence showing a satiety effect of milk and certain whey derived peptides (Roberts *et al.*, 2002; Hall *et al.*, 2003), suggest that whey supplements might facilitate achieving a favorable body weight and composition (Belobrajidic *et al.*, 2004). Data showed that even with moderate protein diets based on whey protein, growing rats show a reduction in growth rate and final body weight, compared to rats fed an equivalent casein diet (Belobrajidic *et al.*, 2004; Badger *et al.*, 2001; Hakkak *et al.*, 2000; Minehira *et al.*, 2000).

The amino acids in the whey fraction of dairy products (e.g. Branched chain amino acids specially leucine) have been hypothesized to decrease adiposity through the partitioning of dietary energy (Layman, 2002) and anabolic effects on muscle (Ha and Zemel, 2003). The data showed that isocaloric substitution of protein for carbohydrate, as in Papakonstantinou study (Papakonstantinou *et al.*, 2003), augments weight and fat loss, while sparing lean body mass in subjects on an energy-restricted diet (Teegarden and Zemel, 2003; Layman, 2002). Further, in the study of Papakonstantinou *et al.* (2003), sucrose content of high calcium diet was 104 grams lower than sucrose content of low calcium diet and nonfat dry milk in high calcium diet was 200 g/kg diet against zero in low calcium diet. Sucrose is a dietary factor responsible for diet induced obesity (Wetzler *et al.*, 2003; Morris and Zemel, 2005) and may alter the effects of calcium levels.

Lowering effects of PTH for intracellular calcium and thereby increasing lipogenesis and inhibiting lipolysis (Shi *et al.*, 2001; Zemel *et al.*, 2000; Gunther *et al.*, 2005) has been a suggested mechanism for the effect of calcium on body weight and fat.

Although we did not find significant body weight or fat differences, serum levels of parathyroid hormone in HC group was significantly lower than other groups and a dose related increase in total serum calcium was seen. These results are not in accordance with the hypothesis of parathyroid hormone effects on body fat suggested by (Zemel *et al* ., 2000; Zemel, 2003). In another study on human subjects, in spite of the increase in fasting

serum parathyroid hormone concentrations in high calcium diet versus low calcium diet group, the final weight changes did not show significant differences (0.9±2.5 kg and 0.8±2.5 kg) (Gunther *et al.*, 2005).

High calcium intakes may also cause weight loss, because they bind to fatty acids (saponification) (Papakonstantinou *et al.*, 2003) and bile acids in the gut, leading to fat malabsorption and fecal fat excretion (Govers and Vandermeer, 1993; Denke *et al.*, 1993; Mitchell *et al.*, 1968; Shahkhalili *et al.*, 2001). Our findings of fecal fat excretion also did not show significant differences. Welberg *et al.* (1994) in a study compared the effect of 2 grams elemental calcium with placebo and found 0.6 percent difference in fecal fat excretion in human cases (Welberg *et al.*, 1994). This amount of fat excretion could not be the source of great variation in weight (Sakhaee and Malouf, 2005). However in the study of Papakonstantinou *et al.* (2003) and Jacobson *et al.* (2005) the amounts of fecal fat excretion were of significant importance and could imply the differences in weight gain. Shi *et al.* (2001), Zemel *et al.* (2000) and Sun and Zemel (2004) did not report the amount of fecal fat excretion in their studies. Fat saponification is affected by amounts and types of calcium (e.g. ionized calcium, complex calcium) (Bosworth *et al.*, 1918), intraluminal pH (Ditscheid *et al.*, 2005; Van der Meer and DeVries, 1985; Boyd *et al.*, 1932), fatty acid types (saturated vs. unsaturated) (Graham and Sackman, 1983) and their position in triglycerides (Lopez-Lopez *et al.*, 2001; Allen, 1982; Nelson *et al.*, 1998). Therefore these factors must be accounted while interpreting the findings of these types. The diets used in the experiment of Papakonstantinou *et al.* (2003) and Zemel *et al.* (2000) contained 25% kcal fat, of which 22.7% came from soybean oil and the remainder was from lard. In addition to lower calcium concentration, in our study, the whole content of diet fat was from soybean oil. Soybean oil contains ~11% by weight palmitate and 4% by weight stearate, whereas lard contains ~ 28% palmitate and 14% stearate (Papakonstantinou *et al.*, 2003). Therefore, it is possible that the fecal fat differences between the groups have been the result of different proportions of lard and the saturated fatty acids that have been used in their diets.

Other suggested mechanisms for calcium effects on body weight are unpalatability of the diet and therefore lower intake of high calcium diets and/or increasing satiety (Zhang and Tordoff, 2004). Like Zhang and Tordoff (2004) in our study total food intake in HC diet group was insignificantly lower than RC and LC group. In spite of differences in weight changes, Shi *et al.* (2001), Zemel *et al.* (2000), Sun and Zemel (2004) and Papakonstantinou *et al.* (2003), in their studies, reported insignificant differences in food intake between different calcium levels. Briefly, the data do not support effects of calcium on food palatability and intake.

In conclusion, our results show that dietary calcium had no lowering effect on body fat and weight in normal physiologic rats. Further dietary calcium did not affect the amount of food intake or satiety. More experimental studies and clinical trials are necessary for comparing the effects of dairy and elemental sources of calcium through fecal fat excretion, using different levels of calcium, while controlling other components of milk, including peptides and amino acids.

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