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Effect of high protein diet on stone-forming propensity and bone loss in rats

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Background. High protein diets are believed to cause kidney stone formation and bone loss, but the mechanisms mediating these changes are unknown. The purpose of this study was to create an animal model of animal protein excess and to evaluate the response of kidney and bone to the dietary protein load.

Methods. Rats (12 per group) were pair-fed with a high (48%) and low (12%) casein diets that were otherwise identical in their content of sodium, potassium, calcium, phosphorus, and magnesium.

Results. Compared with the low casein group, the high casein group delivered a substantial acid load during 59 days of study, since it significantly decreased urinary pH, and increased urinary ammonium, titratable acidity, and net acid excretion. Animals on high casein diet also had higher urinary volumes. On the high casein diet, urinary calcium excretion was significantly higher and urinary citrate excretion and concentration was significantly lower. On the high casein diet, urinary saturation of calcium phosphate was higher. Serum calcitriol concentration did not significantly differ between the two groups. Histomorphometric analysis of femur procured after 59 days on the diet showed marked increase in bone resorption in the high casein group. Hypocitraturia was associated with increased activity of sodium-citrate cotransporter in renal cortical brush-border membranes (BBM) in the high casein group.

Conclusion. Both the kidney and bone contribute to the pathogenesis of hypercalciuria during high casein diet in rats. Hypocitraturia is probably renal in origin. This rat model will be useful in elucidating the mechanisms by which high protein intake increases the risk of nephrolithiasis and bone loss in human beings.

The association of animal protein consumption with kidney stone formation has long been recognized. A risk analysis revealed that animal protein consumption carries increased risk for stone formation [1]. A recent randomized trial disclosed that that a diet restricted in animal proteins and sodium was effective in controlling recurrent formation of calcium oxalate stones among patients with hypercalciuria [2].

Two major factors cited for stone formation from animal protein excess are hypocitraturia [3–6] and hypercalciuria [4, 6]. Hypocitraturia has been ascribed to the acid load conferred by the high animal protein diet [5, 6]. Many schemes have been implicated for the development of hypercalciuria from ingestion of animal proteins spanning the three principal organs of calcium homeostasis [7]. In the kidney, these include direct acid-mediated impairment of renal tubular reabsorption of calcium [8], altered renal handling of calcium from stimulation of prostaglandin synthesis [9], changes in membrane phospholipid content [10], and hyperfiltration of calcium from increased renal parenchymal mass [11]. In the bowel, enhanced calcium absorption has been ascribed to stimulated calcitriol synthesis from enlarged renal mass [11]. Another potential complication of high dietary intake of animal proteins is bone loss. Bone loss has been attributed to elaborating bone-resorbing cytokines [12]. Moreover, acid load from animal protein excess may stimulate osteoclastic bone resorption, and inhibit osteoblastic collagen synthesis [13].

We recently investigated metabolic effects of a weight-reducing, high animal protein-low carbohydrate diet on stone-forming propensity and bone metabolism in 10 normal subjects [6]. This diet delivered an acid load of about 50 mEq/day, reduced urinary citrate by 200 to 300 mg/day, and increased urinary calcium by 90 to 100 mg/day. The urinary saturation of calcium oxalate increased by about 35% and the estimated calcium balance turned less positive during the high protein diet, it was difficult in human beings to ascertain the exact causes of hypercalciuria and hypocitraturia, or to assess the state of bone turnover.

We therefore sought to create an animal model of animal protein excess in rats. If successful, the animal model will allow us to conduct appropriate studies to

Key words: animal protein, nephrolithiasis, hypercalciuria, hypocitraturia, bone loss.

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Table 1. Composition of high and low casein diets

Constituents	Low Casein 12% g/kg	High Casein 48% g/kg
Casein	138	552
DL-Methionine	2.1	0
Corn starch	453	51
Sucrose	120	120
Maltodextrin	120	120
Soybean oil	66	63
Mineral mix, Ca-P deficient (TD 79055)	13.37	13.37
Choline bitartrate	2.5	2.5
Calcium phosphate, dibasic ($CaHPO_4$)	13.71	1.011
Calcium carbonate	8.225	17.37
Sodium chloride	9.807	9.743
Potassium phosphate, dibasic (K_2HPO_4)	1.838	1.813
Ferrous sulfate, $FeSO_4$ - 7 H_2O	0.325	0.315
Magnesium oxide, MgO	0.451	0.43
Kcal/100 g diet	371.44	369.22
% fat (by weight)	6.83	6.81
% calcium	0.7355	0.7356
% phosphorus	0.4413	0.4414
% sodium	0.4882	0.4882
% potassium	0.4629	0.4628
Magnesium *ppm*	782.1	781.8

unveil the underlying cellular-molecular basis for hypocitraturia, hypercalciuria, stone-forming propensity, and bone loss. In this communication, we describe studies in rats fed a high casein diet as a model of animal protein excess. These rats displayed marked hypercalciuria, hypocitraturia, increased urinary saturation of calcium phosphate, and pronounced bone loss on histomorphometric analysis.

METHODS

Experimental protocol

Thirty-six (Sprague-Dawley) male rats, (8 weeks old, average weight 232 g) (Charles River, Boston, MA, USA) were randomized into two groups of 18 rats each receiving either low casein or high casein diets. The diets were matched for sodium, potassium, phosphorus, calcium, and magnesium (Table 1). The main difference between the two diets was in the sulfur content (1.57 g per kg of low casein chow, and 3.34 g per kg of high casein chow).

After 4 days of feeding (days 1 to 4) with high (48%) or low (12%) casein diets, 12 rats from each group were transferred to metabolic cages for pair feeding and urine collection for 55 days (days 5 to 59). Rats were housed individually. The high casein group was fed ad libitum but the amount of food offered to the low casein group was limited to the average intake of high casein group from the previous day. Food was provided at 10:00 a.m. each day. All animals were given distilled water ad libitum for the duration of the study. Weight and urine volume were recorded for all animals on each day of urine collection.

Urine collection for biochemical analysis. On days 11 and 12, 19 and 26, 40 and 47, and 55 and 59, a 24-hour urine sample from each animal was collected under 3 mL of mineral oil and 5 crystals of thymol. Urine samples were transferred into graduated centrifuge tubes and urine volumes were recorded.

Biochemical analysis in serum, and studies in kidney tissue. On day 60 (after 59 days on the respective diets), all 24 rats (12 on low casein and 12 on high casein diets) in metabolic cages were sacrificed. Blood was drawn from the inferior vena cava for biochemical analysis. Kidneys were harvested for sodium citrate cotransporter (NaDC-1) activity in brush-border membranes (BBM).

Harvesting of bone. After 4 days of feeding with high (48%) or low (12%) casein diet, the remaining six rats from each group were pair-fed according to the abovementioned protocol for 55 days. A 70% ethanol solution containing 300 mg tetracycline/mL was prepared and was kept in a glass tube wrapped in aluminum in the dark. Prior to use it was diluted 1:10 in a saline solution buffered with phosphate to pH 7.4 resulting in a working solution of 30 mg tetracycline/mL. Rats were injected intraperitoneally with tetracycline (30 mg per kg body weight) on days 45 and 46, and again on days 55 and 56. On day 60 (after completion of 59 days on the diet), animals were sacrificed. Femora were harvested and stored for bone histomorphometric analysis.

BBM isolation. After the kidneys were removed, they were placed in an iced-cold buffer solution (pH 7.5) (in mmol/L) [300 mannitol, 5 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 0.1 phenylmethyl sulfonylfluoride, and 18 tris(hydroxymethyl) aminomethane (Tris)]. Thin superficial cortical slices were homogenized with a polytron homogenizer, and the BBM vesicles were isolated by differential centrifugation and magnesium precipitation as previously described [18]. The final BBM fraction was washed twice, and the final pellet was resuspended in a buffer solution (pH 7.5) (in mmol/L) [200 mannitol, 50 KCl, 6 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), and 10 Tris].

Analytic methods

Biochemical analysis. Urinary sodium and potassium were analyzed by flame photometry, and calcium and magnesium by atomic absorption spectrophotometry. Phosphorus, creatinine, ammonium and citrate were measured by autoanalyzer (Cobas MIRA) (Roche Diagnostics, Inc., Montclair, NJ, USA). Oxalate and sulfate were determined by ion chromatography (Dionex 2001) (Dionex, Sunnydale, CA, USA). Titratable acidity was determined by titrating the urine sample to pH 7.4

with 0.1 N sodium hydroxide. Serum chemistries were obtained by using an autoanalyzer. Serum parathyroid hormone (PTH) as assayed with immunoradiometric assay (IRMA) kit (Nichols Institute, San Juan Capistrano, CA, USA). Serum 1,25-$(OH)_2$ vitamin D (calcitriol) was analyzed by the method previously described [14].

Net acid excretion was obtained (in mEq/day) as [titratable acidity + ammonium] – [bicarbonate – citrate$^{2-/3-}$]. Urinary citrate was included in this equation because citrate$^{2-/3-}$ is a legitimate urinary base. Urinary saturation of calcium oxalate and brushite ($CaHPO_4 \cdot 2\,H_2O$) was estimated from relative saturation ratio (RSR), or the ratio of activity product of calcium oxalate or brushite and the respective thermodynamic solubility product [15]. A ratio of 1 indicated saturation, >1 supersaturation, and <1 undersaturation.

Histomorphometric analysis. Bone histomorphometry was performed on the distal metaphysis of the left femur from each rat after double tetracycline labeling. After removing the entire femur, the distal half was placed in Villanueva osteochrome (Polysciences, Warrington, PA, USA) for 72 hours. The specimen was then dehydrated in a graded series of alcohol and processed undecalcified in methylmethacrylate as previously described [16]. Ten micron sections were obtained in the longitudinal plane. The histomorphometric examination was performed at the computer monitor on images captured from an Aus Jena microscope with an Optiplex video camera attachment. The bone histomorphometry software (Bioquant Nova II) (R&M Biometrics, Nashville, TN, USA) permitted quantitation of areas, lengths, and individual distances. Static measurements of cancellous bone volume and thickness and cellular parameters were made on toluidine blue–stained sections. Fluorochrome-based indices of bone formation were measured in unstained sections. Measurements of cancellous bone were taken at a distance of at least 1 mm from the growth plate to prevent inclusion of the primary spongiosa. The terminology used is that recommended by the histomorphometry nomenclature committee of the American Society for Bone and Mineral Research [17].

BBM citrate transport measurements. Citrate transport activity measurements were performed in freshly isolated BBM vesicles by the radiotracer uptake using the rapid Millipore rapid filtration technique (Millipore, Bedford, MA, USA) as previously described [19, 20]. Uptake was initiated by vortex mixing 100 μg protein of BBM vesicles preloaded with intravesicular buffer (in mmol/L) (200 mannitol, 50 KCl, 16 Hepes, and 10 Tris, pH 7.5) with a 5 volume excess of uptake solution (in mmol/L) 100 NaCl, 50 KCl, 16 Hepes, 10 Tris, pH 7.50, and 0.1 ^{14}C-citrate). For Na^+-independent uptake, NaCl in the uptake solution was replaced by choline chloride. Transport was terminated by ice-cold stop solution (in mmol/L) (135 NaCl, 10 Na succinate, 16 Hepes, and 10 Tris, pH 7.5) and then rapidly filtered through a presoaked 0.65 μm pore-size cellulose nitrate filter (DAWP 02500) (Millipore) under vacuum suction. Filter blanks were determined similarly by adding the ice-cold stop solution to BBM vesicles simultaneously with the uptake solution. All uptake measurements were performed at 25°C in triplicate, and uptake was calculated as picomoles citrate per time interval per milligram BBM protein.

Statistical analysis

Differences between the high and low casein diets were assessed using two-factor repeated measures analysis of variance (ANOVA) for urinary determinants of acid-base status and stone risk factors. A between group factor (two casein groups) and a repeated factor (different study days) were included in the model. Comparisons of two diets at each time period were derived from the analysis of variance model. Significant difference in histomorphometric indices between the two diets was assessed by Wilcoxon rank sum test. Significant difference between the two diets in serum biochemistry, endogenous creatinine clearance and BBM transport was assessed by Student *t* test. Results are presented as mean and standard deviation. Statistical analysis was performed with SAS version 8.2 (SAS Institute, Cary, NC, USA).

RESULTS

Diet consumption, body weight, and urine collection

During 59 days of study in metabolic cages, food consumption gradually declined in both groups ($N = 12$ in each group). In the high casein group, mean food consumption was 21.7 g/day on days 11 and 12, 20.0 g/day on days 19 and 26, 17.6 g/day during days 40 and 47, and 15.6 g/day on days 55 and 59. In the low casein group, mean food consumption was 22.0 g/day during days 11 and 12, 20.5 g/day during days 19 and 26, 16.5 g/day during days 40 and 47, and 15.4 g/day during days 55 and 59.

After 4 days on casein diets, average body weight before pair-feeding was 236 g in the high casein group and 231 g in the low casein group. In both diet groups, body weight gradually increased during the study. In the high casein group, mean body weight was 293 g on days 11 and 12, 352 g on days 19 and 26, 430 g on days 40 and 47, and 444 g on days 55 and 59. In the low casein group, mean body weight was 276 g on days 11 and 12, 339 g on days 19 and 26, 402 g on days 40 and 47, and 404 g on days 55 and 59. There is a trend for higher body weight in the high casein group but the differences between the two diet groups at corresponding time periods were not statistically significant.

Twenty-four–hour urine collections were made at the indicated times. Urine volumes were higher in the high casein group at all time points (Table 4). Total urinary

Table 2. Measures of acid-base status during low and high casein diets

	Days on the diet			
	11 and 12	19 and 26	40 and 47	55 and 59
Urinary pH				
Low casein	6.97 ± 0.36	7.34 ± 0.47	6.92 ± 0.19	7.34 ± 0.16
High casein	6.14 ± 0.22[a]	6.25 ± 0.22[a]	6.21 ± 0.13[a]	6.24 ± 0.13[a]
Urinary sulfate *mmol/day*				
Low casein	0.28 ± 0.04	0.29 ± 0.03	0.36 ± 0.07	0.20 ± 0.04
High casein	1.54 ± 0.35[a]	1.59 ± 0.30[a]	1.69 ± 0.19[a]	1.36 ± 0.33[a]
Urinary ammonium *mEq/day*				
Low casein	0.50 ± 0.14	0.84 ± 0.47	0.58 ± 0.11	0.56 ± 0.47
High casein	2.71 ± 0.70[a]	2.81 ± 0.77[a]	2.90 ± 0.36[a]	2.32 ± 0.42[a]
Titratable acidity *mEq/day*				
Low casein	0.04 ± 0.04	0.01 ± 0.01	0.06 ± 0.04	0.02 ± 0.01
High casein	0.51 ± 0.21[a]	0.68 ± 0.20[a]	0.64 ± 0.15[a]	0.52 ± 0.17[a]
Net acid excretion *mEq/day*				
Low casein	−0.14 ± 0.19	−0.41 ± 1.27	0.25 ± 0.18	0.24 ± 0.45
High casein	2.99 ± 0.87[a]	3.29 ± 0.74[a]	3.43 ± 0.39[a]	2.77 ± 0.52[a]

All values are expressed as mean ± SD.
[a] $P < 0.001$ for significant difference between the two diets. In this table as well as in Table 4, the average of two determinations obtained during 2 days of each time period was taken for each rat in calculating the group mean.

Table 3. Serum biochemistry during low and high casein diets on day 60

	Low casein	High casein
Serum		
Sodium *mEq/L*	135 ± 2	138 ± 1
Potassium *mEq/L*	6.95 ± 1.18	5.53 ± 0.38[a]
Chloride *mEq/L*	99 ± 1	101 ± 1
Bicarbonate *mEq/L*	22.8 ± 1.7	23.6 ± 2.4
Calcium *mg/dL*(mmol/L)	9.8 ± 0.3 (2.45 ± 0.07)	9.7 ± 0.3 (2.42 ± 0.07)
Phosphorus *mg/dL* (mol/L)	7.5 ± 0.7 (2.42 ± 0.23)	7.1 ± 0.3 (2.29 ± 0.10)
PTH *pg/mL*	76 ± 57	44 ± 17
Calcitriol *ng/mL*	60 ± 22	52 ± 12

PTH is parathyroid hormone.
All values are expressed as mean ± SD. Serum electrolytes, calcium, and phosphorus were determined in 12 rats from each group. However, serum PTH and calcitriol could be analyzed in only 10 rats on low casein diet, and 9 rats on high casein diet.
[a] $P < 0.01$ between the two diets.

creatinine was higher in the casein group on days 19 through 59 (not shown). Creatinine normalized per body weight was numerically higher in the high casein group but was not significantly different between the two groups (low casein vs. high casein in mg/kg, mean ± SE): 29.7 ± 2.9 vs. 31.7 ± 7.2 on days 11 and 12; 24.2 ± 2.4 vs. 28.1 ± 2.5 on days 19 and 26; 30.8 ± 2.5 vs. 36.1 ± 4.0 on days 40 and 47; and 23.8 ± 4.7 vs. 30.4 ± 2.9 on days 55 and 59.

Acid-base parameters

The effect of high casein diet on indices of acid-base balance is shown in Table 2. Urinary pH was significantly lower, and urinary sulfate, ammonium, and titratable acidity were significantly higher on high casein diet compared with the low casein diet. Net acid excretion (NAE) was low on the low casein diet. On the high casein diet, it was higher by 2.5-fold to 3.7-fold (Table 2). Note that the increase in NAE (∼3 mEq/day) exceeded the increase in urinary sulfate excretion (∼2.4 mEq/day) induced by the high casein diet. Urinary bicarbonate was (mean ± SE in mEq/day low casein vs. high casein) 0.15 ± 0.12 vs. 0.05 ± 0.03 (days 11 and 12), 0.90 ± 1.56 vs. 0.07 ± 0.10 (days 19 and 26), 0.10 ± 0.10 vs. 0.04 ± 0.01 (days 40 and 47), and 0.20 ± 0.09 vs. 0.03 ± 0.01 (days 55 and 59). Serum sodium, chloride, bicarbonate, and anion gap obtained on the day of sacrifice (day 60 after 59 days on the diet) did not differ significantly between high and low casein diets (Table 3). However, serum potassium was significantly lower on the high casein diet.

Stone risk factors

Key urinary stone risk factors are presented in Tables 4 and 5. On both diets, each stone risk factor tended to decline as the study progressed. However, at each time period, urinary excretion of calcium, phosphorus, and magnesium were significantly higher, and urinary citrate was significantly lower, during the high casein diet than on the low casein diet (Table 4). Throughout the study, urinary calcium excretion remained 2.7-fold to 3.6-fold higher in the high casein group (Fig. 1 and Table 4). Because of the higher urinary volume, urinary calcium concentration was not significantly higher on the high casein diet (Table 5). Urinary citrate excretion in the high casein group was 61% to 78% lower than in the low casein group (Fig. 1 and Table 4). Urinary citrate concentration in the high casein group was only 11% of that of the low casein group (Table 5). Urinary phosphate excretion was 5- to 14-fold higher (Table 4) and urinary phosphate concentration was 2- to 6-fold higher (Table 5) in the high casein group. In contrast, there was no significant difference in urinary oxalate excretion (Table 4). There was no difference in sodium, or potassium excretion between the two diets (not shown).

When saturations were analyzed using concentrations (Table 5), calcium oxalate urinary saturation remained relatively stable and was not higher in the high casein diet. In fact, on days 55 and 59, calcium oxalate was actually slightly but significantly lower in the high casein group. The saturation of brushite was constant and remained consistently higher in the high casein group (Table 5).

Indices of calcium metabolism and creatinine clearance

There was no significant difference in serum calcium, phosphorus, PTH, and calcitriol between high and low casein diets (Table 3). However, endogenous creatinine clearance was significantly higher in the high casein group (0.015 mL/min vs. 0.026 mL/min, $P < 0.01$).

Histomorphometric findings

Bone histomorphometric findings are summarized in Table 6. There were no significant differences between

Table 4. Urinary stone risk factors: Low vs. high casein diets (excretion rates)

	Days on the diet			
	11 and 12	19 and 26	40 and 47	55 and 59
Calcium *mg/day* (mmol/day)				
Low casein	1.00 ± 0.83 (0.025 ± 0.021)	0.76 ± 0.41 (0.019 ± 0.010)	0.74 ± 0.42 (0.018 ± 0.010)	0.63 ± 0.37 (0.016 ± 0.009)
High casein	3.59 ± 1.83[a] (0.090 ± 0.046)	3.05 ± 1.58[a] (0.076 ± 0.039)	1.98 ± 0.98[b] (0.049 ± 0.024)	1.95 ± 0.90[b] (0.049 ± 0.022)
Oxalate *mg/day* (μmol/day)				
Low casein	0.52 ± 0.11 (5.8 ± 1.2)	0.58 ± 0.10 (6.4 ± 1.1)	0.50 ± 0.09 (5.6 ± 1.0)	0.36 ± 0.10 (4.0 ± 1.1)
High casein	0.49 ± 0.12 (5.4 ± 1.3)	0.45 ± 0.07 (5.0 ± 0.8)	0.39 ± 0.13 (4.3 ± 1.4)	0.30 ± 0.06 (3.3 ± 0.7)
Phosphorus *mg/day* (mmol/day)				
Low casein	4.0 ± 1.7 (0.13 ± 0.05)	1.4 ± 1.0 (0.05 ± 0.03)	4.6 ± 1.7 (0.15 ± 0.05)	2.4 ± 0.9 (0.08 ± 0.03)
High casein	21.2 ± 6.3[a] (0.68 ± 0.20)	24.3 ± 6.0[a] (0.78 ± 0.19)	22.0 ± 3.4[a] (0.71 ± 0.11)	17.4 ± 4.2[a] (0.56 ± 0.14)
Citrate *mg/day* (mmol/day)				
Low casein	48.8 ± 12.4 (0.25 ± 0.06)	32.6 ± 6.2 (0.17 ± 0.03)	26.5 ± 8.0 (0.14 ± 0.04)	12.4 ± 5.7 (0.06 ± 0.03)
High casein	15.2 ± 6.4[a] (0.08 ± 0.03)	10.3 ± 5.2[a] (0.05 ± 0.03)	5.8 ± 2.5[a] (0.03 ± 0.01)	3.6 ± 1.1[b] (0.02 ± 0.01)
Magnesium *mg/day* (mmol/day)				
Low casein	5.10 ± 1.08 (0.210 ± 0.044)	3.45 ± 1.33 (0.142 ± 0.055)	2.52 ± 0.92 (0.104 ± 0.038)	1.70 ± 0.89 (0.070 ± 0.037)
High casein	6.84 ± 1.61[a] (0.281 ± 0.066)	7.00 ± 1.48[a] (0.288 ± 0.061)	6.48 ± 0.98[a] (0.267 ± 0.040)	4.79 ± 0.94[a] (0.197 ± 0.039)
Urinary volume *mL/day*				
Low casein	11 ± 3	12 ± 4	10 ± 2	8 ± 3
High casein	33 ± 9[c]	29 ± 7[c]	26 ± 4[c]	20 ± 4[b]

All values are expressed as mean ± SD.
[a]$P < 0.001$; [b]$P < 0.01$; [c]$P < 0.05$ between two diets. The mean value of 18 mL for all samples from all rats was taken in calculating relative saturation ratio (RSR).

the high and low casein groups for any of the bone histomorphometric parameters except for the measurements of bone resorption (BS), namely eroded surface (ES) and osteoclastic surface (OcS). There were marked and significant increases in both of these parameters for the rats on the high casein diet. High casein diet increased eroded surface (ES/BS) threefold (6.5% ± 1.22% vs. 19.6% ± 1.79%, $P < 0.001$) and osteoclastic surface (OcS/BS) 3.3-fold (2.3% ± 0.48% vs. 7.7% ± 0.97%, $P < 0.01$). Mean values for osteoid indices (osteoid volume, osteoid surface, and osteoblastic surface) were not significantly different.

BBM NaDC-1 activity

BBM transport of citrate in the high casein group was higher than in the low casein group (213 vs. 133 pmol/second/mg BBM protein, $P < 0.01$) (Fig. 2). BBM transport of sulfate, serving as the control for citrate transport, showed no significant difference between high and low casein groups ($P = 0.8$).

DISCUSSION

This study was undertaken in order to determine if a high casein diet in rats could serve as a model to study the effect of high animal protein diet on mineral metabolism. Compared to rats kept on a low casein diet, rats on a high casein-fed showed a much higher net acid excretion, urinary calcium and phosphate, and a much lower urinary citrate excretion rate. Urinary saturation of calcium oxalate did not increase due to the higher urinary volumes in the high casein diet. Brushite saturation increased markedly throughout the whole study. Urinary citrate excretion and concentration were both markedly reduced. No kidney stones or nephrocalcinosis were noted by gross examination in this group of animals.

Note that the high casein diet-induced increase in urinary phosphate exceeded that of calcium consistently in all time points. Both bone phosphate release and acid-induced renal phosphaturia can account for some of the phosphaturia. However, it is more likely that there is more organic phosphorus in the high casein diet. This is compatible with the fact that the high casein-induced increment in NAE (mean $\Delta = 3.13$ mEq/day) exceeded the increment in sulfate excretion (mean $\Delta = 2.52$ mEq/day), suggesting that part of the acid load in the high casein diet was derived from nonsulfur containing amino acids such as phosphoserines and phosphothreonines.

Rats on the high casein diet had much higher urinary volumes. The etiology of this is not established but may be

Table 5. Urinary stone risk factors: Low vs. high casein diets (concentrations)

	Days on the diet			
	11 and 12	19 and 26	40 and 47	55 and 59
Calcium *mg/L* (mmol/L)				
Low casein	92 ± 81 (2.3 ± 2.0)	65 ± 34 (1.6 ± 0.8)	74 ± 31 (1.9 ± 0.8)	79 ± 21 (2.0 ± 0.5)
High casein	108 ± 45 (2.7 ± 1.1)	101 ± 46 (2.5 ± 1.1)	77 ± 37 (1.9 ± 0.9)	97 ± 35 (2.4 ± 0.9)
Oxalate *mg/L* (μmol/L)				
Low casein	48 ± 13 (0.53 ± 0.14)	52 ± 16 (0.58 ± 0.17)	54 ± 16 (0.60 ± 0.17)	52 ± 23 (0.58 ± 0.26)
High casein	15 ± 3[a] (0.17 ± 0.03)	16 ± 3[a] (0.18 ± 0.04)	15 ± 5[a] (0.17 ± 0.06)	16 ± 3[a] (0.17 ± 0.03)
Phosphorus *mg/L* (mmol/L)				
Low casein	369 ± 199 (11.9 ± 6.4)	137 ± 144 (4.4 ± 4.7)	487 ± 160 (15.7 ± 5.2)	340 ± 144 (11.0 ± 4.6)
High casein	641 ± 124[a] (20.7 ± 4.0)	828 ± 120[a] (26.7 ± 3.9)	873 ± 161[a] (28.2 ± 5.2)	893 ± 151[a] (28.8 ± 4.9)
Citrate *mg/L* (mmol/L)				
Low casein	4473 ± 1380 (23.5 ± 7.3)	2964 ± 1148 (15.6 ± 6.0)	2818 ± 948 (14.8 ± 5.0)	1712 ± 758 (9.0 ± 4.0)
High casein	479 ± 199[a] (2.5 ± 1.0)	356 ± 158[a] (1.9 ± 0.8)	237 ± 124[a] (1.2 ± 0.7)	185 ± 57[a] (1.0 ± 0.3)
Magnesium *mg/L* (mmol/L)				
Low casein	467 ± 129 (19.2 ± 5.3)	315 ± 161 (12.9 ± 6.6)	255 ± 67 (10.5 ± 2.8)	219 ± 91 (9.0 ± 3.7)
High casein	213 ± 55[a] (8.8 ± 2.3)	242 ± 47 (10.0 ± 1.9)	260 ± 60 (10.7 ± 2.5)	219 ± 91 (9.0 ± 3.7)
RSR, calcium oxalate				
Low casein	2.09 ± 1.67	2.27 ± 1.11	2.42 ± 1.10	3.54 ± 1.23
High casein	2.71 ± 1.01	2.40 ± 1.02	1.77 ± 1.01	2.24 ± 0.81[b]
RSR, brushite				
Low casein	0.50 ± 0.57	0.16 ± 0.09	0.60 ± 0.37	0.74 ± 0.40
High casein	1.62 ± 0.61[a]	2.07 ± 0.85[a]	1.70 ± 0.91[a]	2.23 ± 0.88[a]

RSR is relative saturation ratio. All values are expressed as mean ± SD.
[a] $P < 0.001$; [b] $P < 0.01$ between two diets.

secondary to a higher osmolar load from the high protein diet driving higher urine volumes and stimulating thirst. Unfortunately, urinary urea and osmoles were not available from this set of data to confirm this hypothesis. It is unknown whether a higher urine volume accompanies a high protein diet in humans, particularly when the protein load is of much lower magnitude than the current study in rats.

In a recent study [6], human beings maintained on a weight-reducing, high protein low carbohydrate diet revealed qualitatively similar but quantitatively less prominent changes. In human beings kept on a high protein low carbohydrate diet with a mean body weight of 81 kg [6], net acid excretion increased by about 50 mEq/day. In this study of rats weighing between 240 to 440 g, net acid excretion increased by 2.5 to 3.7 mEq/day on the high casein diet. In human subjects kept on a high protein low carbohydrate diet [6], net acid excretion increased by 80% to 90%. In this study, net acid excretion was 2.5-fold to 3.7-fold higher among rats fed a high casein diet than on the low casein diet. The most likely explanation for the different responses between human beings and rats is the different magnitude of acid load delivered.

From the standpoint of their pathogenetic role in stone formation and bone loss, key biochemical disturbances produced by a high casein or animal protein diet are hypocitraturia and hypercalciuria. Hypocitraturia can predispose to the formation of calcium-containing stones since citrate is one of the well-recognized inhibitors of the crystallization of calcium oxalate and calcium phosphate [21]. Hypercalciuria contributes to stone formation by increasing urinary saturation of calcium salts [22] and by binding negatively charged inhibitors of stone formation [23]. Hypercalciuria partially results from bone loss. Bone loss occurred during a high casein diet, as evident by marked increase in bone resorption on histomorphometric analysis. Although numerically, there was some trend toward lower bone formation, none of the difference formation parameters were statistically significant. Note that these animals continued to grow despite the high acid load and bone abnormalities compared to young humans with renal tubular acidosis who have stunted growth. One possible explanation is that the high acid load from dietary protein is within the acid-excreting capacity of the kidney resulting in preserved plasma acid-base parameters. In contrast, individuals with renal tubular acidosis have impaired renal acidification and abnormal plasma pH and bicarbonate.

There is general agreement that hypocitraturia is renal in origin, attributable to the acid load conferred by the animal protein diet. Clinically, hypocitraturia has been encountered in acquired metabolic acidosis of chronic diarrheal syndrome [24], distal renal tubular acidosis [25], and dietary animal protein consumption [3]. Hypocitraturia is readily ameliorated by administration of potassium alkali [25]. At the cellular level, proximal tubule reabsorption of citrate is coupled to sodium, mediated by the NaDC-1 cotransporter. Reabsorbed citrate is then metabolized within cytoplasm via adenosine triphosphate (ATP)-citrate lyase [26] or within mitochondria via the citric acid cycle. Chronic metabolic acidosis causes hypocitraturia by stimulating the activity of NaDC-1, ATP-citrate lyase and phosphoenolpyruvate carboxykinase (which metabolizes cytoplasmic oxalacetate generated from citrate) [27]. In contrast to the previous studies [26, 27], the acid load in this study was not sufficient to cause detectable systemic acidosis, but yet we found increase NaDC-1 activity in renal BBM in the high casein-fed rats. Hypocitraturia associated with high casein diet is therefore at least in part related to the increased activity of NaDC-1 cotransporter occurring as an adaptive response to the acid load.

Hypercalciuria associated with high casein diet is unlikely to be intestinal in origin. Although intestinal calcium absorption was not directly measured, serum calcitriol concentration was marginally lower on the high casein diet, arguing against the scheme implicating stimulation of calcitriol synthesis from enlarged renal

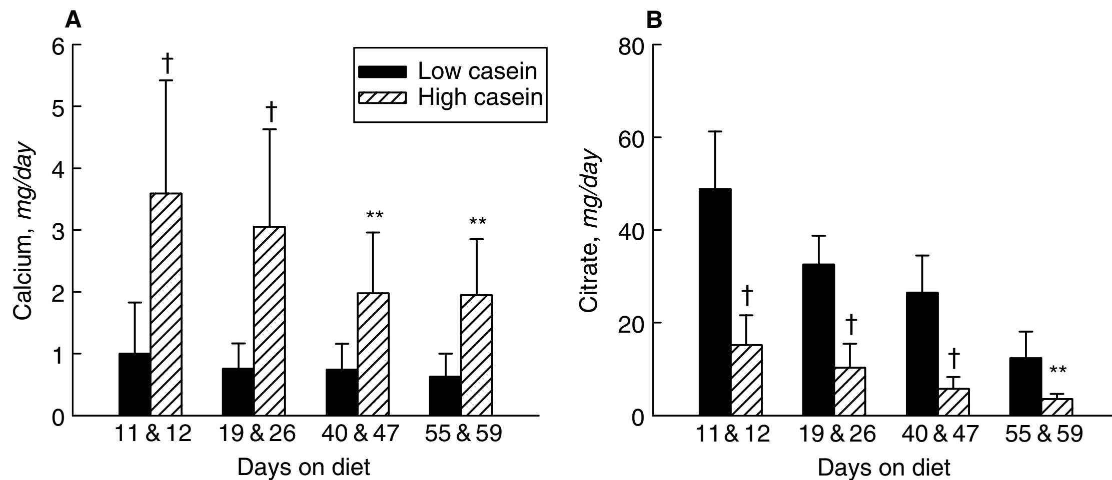

Fig. 1. Comparison of urinary calcium (*A*) and citrate (*B*) between high casein and low casein diets. Bars indicate mean + SD. Significant difference between the two diets is shown by $^{**}P < 0.001$ and $^{\dagger}P < 0.001$.

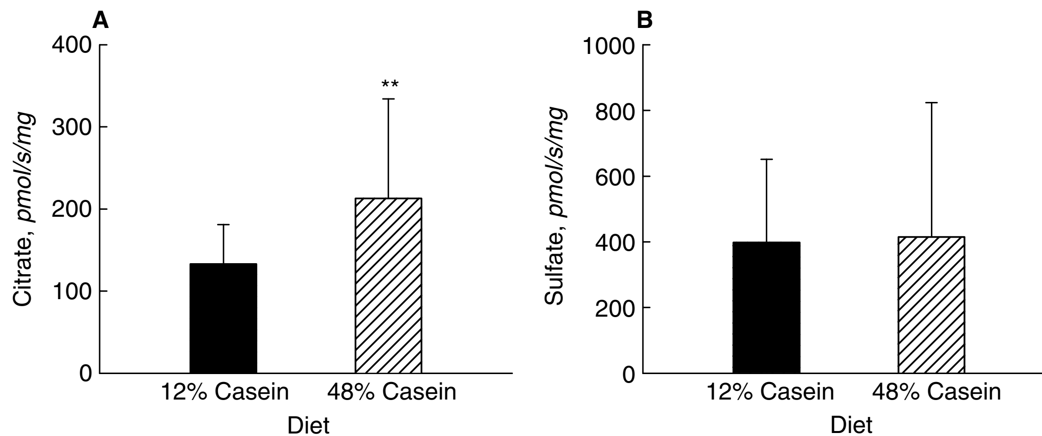

Fig. 2. Citrate (*A*) and sulfate (*B*) transport in brush border basement membranes (BBM) in rats fed low (12%) and high (48%) casein diets. Bars indicate mean + SD. Significant difference is shown by $^{**}P < 0.01$.

Table 6. Bone histomorphometry during low and high casein diets

Histomorphometric index	Low casein diet ($N = 6$)	High casein diet ($N = 6$)
Trabecular thickness μ*m*	53 ± 6	50 ± 3
OV/BV%	1.2 ± 1.0	0.5 ± 0.6
OS/BS%	3.1 ± 1.1	2.4 ± 1.7
ObS/BS%	2.1 ± 1.0	1.6 ± 1.1
ES/BS%	6.5 ± 3.0	19.6 ± 4.4[a]
OcS/BS%	2.3 ± 1.2	7.7 ± 2.4[b]
MS/BS%	9.2 ± 2.2	10.1 ± 1.9
MAR μ*m/day*	1.1 ± 1.4	1.6 ± 1.0

Abbreviations are: OV/BV, osteoid volume/bone volume; OS/BS, osteid surface/bone surface; ObS/BS, osteoblastic surface/bone surface; ES/BS, eroded surface/bone surface; OcS/BS, osteoclastic surface/bone surface; MS/BS, mineralized surface expressed as one-half single-labeled surface plus double-labeled surface/bone surface; MAR, mineral apposition rate.

[a] $P < 0.001$; [b] $P < 0.01$ are significant difference between high and low casein diets. All values expressed as mean ± SD.

parenchymal mass [11]. Bone loss was indicated by a substantial increase in osteoclastic bone resorption by about threefold on histomorphometric analysis. Bone loss must contribute to the pathogenesis of hypercalciuria. It is unclear from this study whether bone loss is directly the result of acid load itself [12], or occurs secondarily from release of bone-resorbing prostaglandins [28] or cytokines [12].

In addition to calcium release from bone, altered renal handling of calcium must also be involved in the development of hypercalciuria from high animal protein diet. Since there was no change in systemic pH or calcium concentration, the filtered load of calcium, estimated from changes in creatinine clearance, was higher in the high casein-fed rats than in low casein-fed rats by 73%. However, urinary calcium excretion was greater by 2.7-fold to 3.6-fold in the high casein group. Clinically, urinary calcium excretion is directly correlated with net acid excretion [29], and treatment with potassium alkali reduces urinary calcium excretion [30–32], commensurate with a reduction in net acid excretion. In dogs, metabolic acidosis has been shown to impair the renal tubular transport of calcium by micropuncture and renal clearance [8]. Exact cellular-molecular mechanisms

of this acid-induced renal calcium leak remain to be elucidated.

It is noteworthy that many of the urinary biochemical factors showed a gradual decline in their daily excretion rates during the 59 days on casein diets. In part, this decline may reflect reduced intake of food. It may also reflect adaptive mechanisms, the nature of which requires elucidation.

CONCLUSION

A high casein diet in rats delivering a three- to fourfold increase in net acid, may serve as an ideal animal model to study cellular-molecular mechanisms for hypocitraturia, hypercalciuria, and bone loss associated with animal protein consumption.

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