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PROTECTIVE EFFECT OF VITAMIN D, OLIGOFRUCTOSE AND LACTOSE AGAINST HYPERCALCIURIA OF FUROSEMIDE DIURETIC THERAPY IN RATS

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Furosemide therapy lowers pathologically raised blood pressure. However, furosemide causes hypercalciuria. This study was designed to determine the diuretic effect of furosemide on bone minerals (Ca, Mg and K) in right tibial bone of male rats and the possible positive impact of vitamin D, oligofructose (FOS), and lactose against furosemide's diuretic effect on these minerals. The experiment was conducted on 30 male albino rats that were divided into 5 groups of similar mean body weights. Groups 1 and 2 were fed on basal diet. Group 3 was fed on basal diet supplemented with 800 mg of vitamin D. Groups 4 and 5 were fed on basal diet containing 10% FOS and 10% lactose, respectively. Groups 2, 3, 4 and 5 were treated with an oral administration of furosemide (15 mg/kg/day). Rats had diet and drinking water provided *ad libitum* for 4 weeks. The results showed that furosemide caused significantly ($p<0.05$) increased urinary excretion of Ca (245%), Mg (155%) and K (363%) compared to the control. Supplementation of vitamin D, FOS and lactose led to a decrease in Ca, Mg and K excretion in urine compared to the furosemide group. Mean Ca level in plasma and tibia was significantly ($p<0.05$) lower in the furosemide group compared to the control one. Supplementation of vitamin D and FOS raised Ca content in tibia to normal levels. We concluded that vitamin D and FOS supplementation has a protective action against hypercalciuria and bone loss of furosemide diuretic therapy in rats.

INTRODUCTION

Skeleton, kidneys, and small intestine are the major organs involved in calcium homeostasis. In the normal human adult, neutral calcium balance is maintained by integrating the absorption of dietary calcium with the losses in sweat and urine. To achieve normal growth, calcium balance must be positive during infancy and puberty [Matkovic *et al*., 1995].

The skeleton is the most-important reserve of calcium and contains 99% of total body calcium. Under normal circumstances, the serum concentration of calcium is maintained within a narrow limit. In addition, and less quickly, bone contributes to maintain calcium homeostasis by coupling the processes of bone formation and bone resorption, which are hormonally modulated [Broadus, 1996]. Accordingly, it is reasonable to hypothesize that chronic disorders of calcium metabolism will secondarily lead to changes in the mineral composition of bone. The relationship between bone mineral status and hypercalciuria is controversial. Long-term therapy of furosemide diuretics cause hypercalciuria and a decrease in bone mineral content [Fernandez *et al.,* 1999].

Atkinson *et al.* [1988] showed that treatment with furosemide diuretics in neonates might be associated with abnormal renal losses of calcium, sodium, chloride and potassium. Condon *et al*. [1999] showed that furosemide may decrease calcium levels by 0.5–1 mmol/L in serum but result in a high renal filtered load of calcium which, if above 3.7 mmol/L in serum, causes calcium phosphate protein complexes, thus giving rise to hypercalcaemic. Much research in experimental animals has shown positive effects of FOS and lactose on Ca and Mg [Hirama *et al.,* 2003; Kruger *et al.,* 2003; Scholz-Aholz *et al.,* 2001]. Vitamin D is necessary for normal growth and has complex effects on bone mineralization [Millonig *et al.,* 2005]. The potential influence of furosemide diuretic effect on bone mineral composition is undetermined. To clarify this, we studied the effect of furosemide diuretic on bone minerals (Ca, Mg and K) in right tibial bone of male rats and the effect of supplementation of three nutrients (vitamin D, FOS, and lactose) to antagonize the hypercalciuria of furosemide diuretic effect.

MATERIALS AND METHODS

Diet. The composition of the basal diet, vitamin mixture [AOAC, 1990] and salt mixture [Müller, 1964] used in this experiment were described in Table 1.

TABLE 1. The composition of the basal diet.

Diet ingredient	Content (g/100 g diet)
Casein	12
Sucrose	10
Corn oil	6
Minerals	5
Vitamins	1
Cellulose	4
Starch	62.0

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Animals. The experiment was conducted on 30 male albino rats weighing 60±2.1 g. The rats were housed in individual stainless steel screen bottom cages and fed on basal diet for one week for adaptation; water was available *ad libitum*. Then, the rats were divided into 5 groups, each of 6 rats of similar mean weights.

Experiment. Groups 1 and 2 were fed on basal diet. Group 3 was fed on basal diet containing 800 mg of vitamin D, group 4 was fed on basal diet containing 10% of fructooligosaccharides (FOS) [Busserolles *et al.,* 2003], whereas group 5 was fed on basal diet +10% lactose. Groups 2, 3, 4 and 5 were administered orally furosemide (15 mg/kg/day) [Koo *et al.,* 1980]. Rats had diet and drinking water provided *ad libitum* for 4 weeks of the experimental period. At the end of the experiment, animals were fasted overnight; blood samples were withdrawn using fine capillary glass tube from the orbital plexus vein. Blood was collected in heparinized tubes and centrifuged at 3000 rpm for 15 min. Plasma samples were stored at -20°C until analysis. The right tibia was excised, cleaned and weighed.

METHODS

Tibial bone was cleaned and dried in an oven at 105°C and accurately weighed, then dry-ashed for 10 h at 550°C [Peterson *et al.,* 1992]. Ash weight was recorded and dissolved in 2 mL of 0.3 mol/L HCl [Pallout *et al*., 1994] prior to analysis for Ca, Mg and K by flame atomic absorption spectrometry. Urinary Ca, Mg and K were determined after mineralization (30 mL of a mixture of concentrated nitric and perchloric acids (1:1) were added to 5 mL of urines and heated in a sand bath until complete disappearance of organic material) using flame atomic absorption spectrometry (Perkin Elmer Atomic Analyst model 300). Dietary content of Ca was determined similarly to bone samples.

In quality control, Standard Reference Material (SRM 1846) consisting of an Infant formulae was obtained from the National Institute of Standards and Technology (Gaithersburg, Maryland, USA) and treated in a similar manner to the unknown bone and food samples. An external standard procedure was adopted throughout the course of the study. The analysis of the SRM 1846 under the present condition gave recoveries of 95.5±2.9, 98.5±2.5 and 97.7±2.7% for Ca, Mg and K, respectively.

Plasma Ca and Mg were determined using kits (Spinreact, Spain). Total bone calcium accumulation in tibia at the end of the 4 weeks was used to measure the relative bioavailability of calcium from food sources according to Ghanem & Hussein [1999]:

Food calcium efficiency = Tibia calcium/Dietary calcium intake (4 weeks) x 100.

Short chain fatty acids were analysed in the caecal content according to Lombard & Dowell [1982] with the use of Hewlett Packard HP 6890 series Gas chromatograph equipped with flame ionization detection (FID) system with ZB-5 (30 m × 53 mm I.D., 50 μm film thickness) column from Phenomenex. Chromatograms of a mixture of short chain fatty acid standards are shown in Figure 2a and those of fecal sample in Figure 2b.

Statistical analysis. Data were expressed as means with standard errors of the mean. One-way analysis of variance (ANOVA) test was used to compare between groups using SPSS version 10 (SPSS Inc., Chicago, USA). A p-value of 0.05 or less was taken to signify statistical significance.

RESULTS

Neither the daily food intake nor the body-weight gain was significantly different between the different experimental groups. The results showed that furosemide dose (15 mg/kg/day) caused significantly increased ($p<0.05$) contents of Ca (245%), Mg (155%) and K (363%) compared to control, and that the supplementation of vitamin D, FOS and lactose led to a significant decrease ($p<0.05$) of the urinary excretion of Ca, Mg and K compared to the furosemide group, as shown in Figure 1a-c.

The efficiency of Ca revealed a significantly lower ($p<0.05$) mean value in the furosemide group compared with the control group (5.5±0.02 *versus* 5.1±0.02 %). The percentage Ca efficiency revealed significantly higher mean values in groups receiving FOS (5.7±0.05) and vitamin D (5.5±0.05) compared with the furosemide group (5.1±0.02%) (Table 2).

The presence of fermentable carbohydrates in the diet (FOS and Lactose groups) resulted in significant ($p<0.05$) enlargement of the caecum and an increase in the concentration of total SCFA compared with the furosemide group (Table 3). The results showed that the levels of propionate (g/kg caecal content) were significantly higher in rats fed on FOS (133±2.33), ($p<0.01$) and lactose (116±2.11) ($p<0.05$) compared to the furosemide group (41.44± 2.74) (Table 3). Non-significant difference was noticed in the concentrations of acetate or valerate in all treated groups compared with the control rats (Table 3, Figure 2).

Mean±SE plasma Ca concentration was 84±4.2 mg/L in the furosemide group and amounted to 139±5.3 and 114.2±7.1 mg/L in both vitamin D and FOS groups, respec-

TABLE 2. Food intake, body weight and calcium efficiency among different study groups.

Groups	Control	Furosemide	Vitamin D	FOS	Lactose
Ca intake (g/4 weeks)	$0.83\pm0.06_a$	$0.84\pm0.02_a$	$0.80\pm0.02_a$	$0.79\pm0.01_a$	$0.82\pm0.02_a$
Initial weight (g)	$58.00\pm3.85_a$	$58.60\pm3.42_a$	$58.0\pm2.45_a$	$59.60\pm3.54_a$	$61.25\pm1.11_a$
Final weight (g)	$105.2\pm6.29_a$	$109.3\pm1.8_a$	$107.6\pm5.18_a$	$102.6\pm5.66_a$	$113.5\pm3.28_a$
Weight gain (g)	$47.2\pm7.33_a$	$50.5\pm2.33_a$	$49.6\pm4.0_a$	$43\pm3.51_a$	$52.25\pm2.49_a$
Food Ca efficiency*	$5.5\pm0.03_a$	$5.1\pm0.02_b$	$5.6\pm0.05_{ac}$	$5.7\pm0.05_{acd}$	$5.2\pm0.04_{abcd}$

a, b, c, d – the same subscripts in the same row indicate no significant differences ($p>0.05$); * Food calcium efficiency= Tibia calcium / Dietary calcium intake (4 weeks) × 100

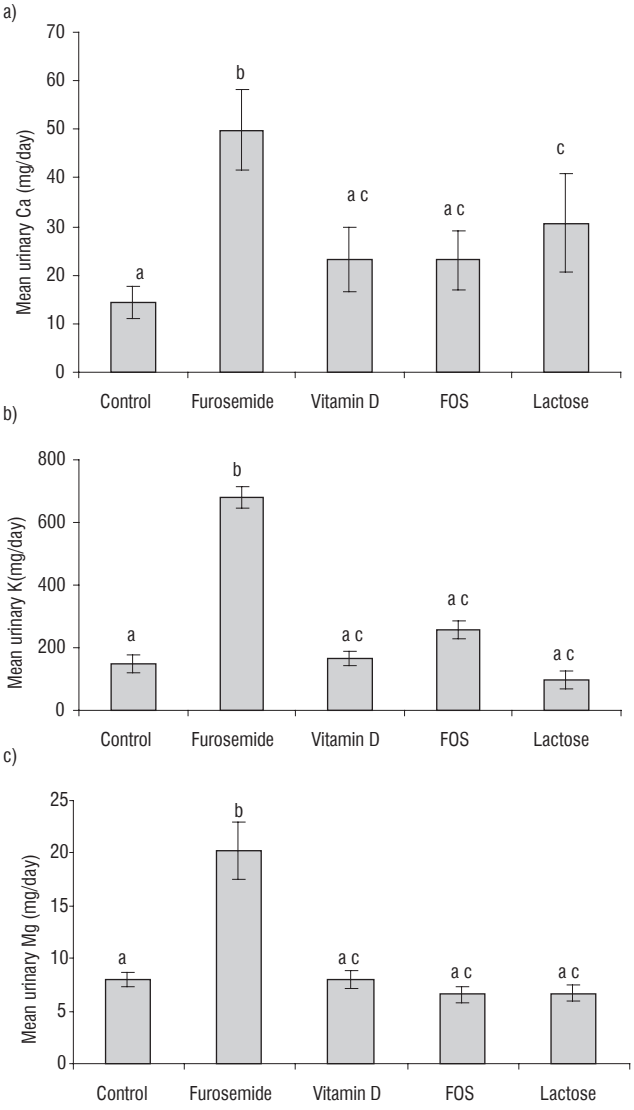

FIGURE 1. Mean urinary excretion of Ca (a), K (b), and Mg (c) (mg/day).

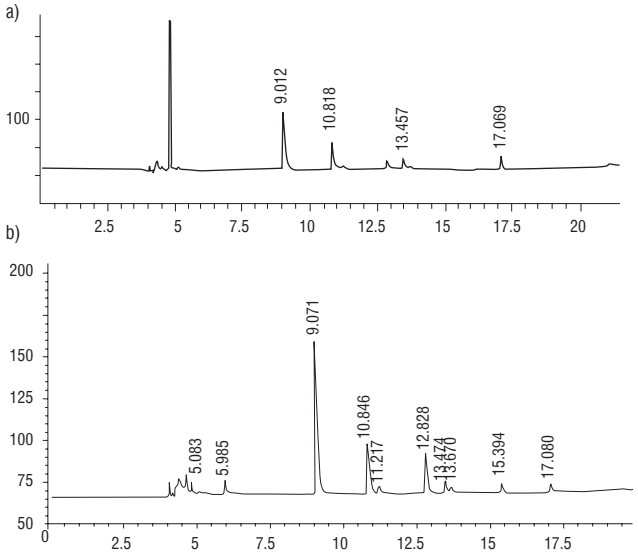

FIGURE 2. Chromatogram of: (a) standards of short chain fatty acids; acetic at 9.01 min; propionic at 10.81; isobutyric at 12.84; butyric at 13.46 and valeric at 17.07 min; and (b) short chain fatty acids of cecal sample; acetic at 9.07 min; propionic at 10.84; isobutyric at 12.84; butyric at 13.47 and valeric at 17.08 min.

tively. A non-significant effect was observed in plasma Mg in the treated groups relative to the furosemide group (Table 4). Also, the non-significant effect was observed in tibial Mg and K in all treated groups as compared to the furosemide group (Table 4).

The results in Table 4 showed a significant decrease in Ca content in the tibia (43.06±0.2 mg/tibia) induced by furosemide (group 2) compared with the control (45.2±0.43 mg). Supplementation of vitamin D or FOS significantly ($p<0.05$) increased mean Ca content in tibia to 44.6±0.36 and 45.1±0.2 mg/tibia in both vitamin D and FOS groups when compared to the furosemide group. The Ca/ash% ratio revealed that Ca represented approximately 37.24% and 40.35% from ash in the group supplemented with vitamin D and FOS compared to furosemide group 34.4%, thus indicating increased mineralization in rats fed supplemented with vitamin D or FOS (Table 4).

DISCUSSION

Furosemide treatment induced hypercalciuria and a lower bone calcium content. The results of the present study showed that, in the animals treated with furosemide, the increased urinary losses of calcium resulted in the stimulation of calcium release from bone. Fernandez *et al.* [1999] reported that furosemide-treatment induced hypercalciuria in rats, lower bone mineral content and bone mineral density. Kim *et al.* [2000] reported that long-term furosemide

TABLE 3. Caecal content and caecal wall weights and short chain fatty acid content among different study groups.

Group	Control	Furosemide	Vitamin D	FOS	Lactose
Caecal content (g)	$1.27\pm0.31_a$	$1.02\pm0.23_a$	$1.32\pm0.39_a$	$2.13\pm0.59_b$	$1.64\pm0.52_{ab}$
Caecal wall weight (g)	$0.25\pm0.05_a$	$0.24\pm0.01_a$	$0.29\pm0.04_a$	$0.45\pm0.05_b$	$0.39\pm0.03_{bc}$
Short Chain Fatty Acids (g/kg caecal content)					
Acetate	$377.0\pm20.33_a$	$367.00\pm5.45_a$	$367\pm5.55_a$	$373\pm20.4_a$	$368\pm10.33_a$
Propionate	$90.3\pm6.61_a$	$41.44\pm2.74_b$	$41.51\pm1.94_b$	$133\pm2.33_c$	$116\pm2.11_d$
Iso-Butyrate	$39.6\pm8.31_a$	$29.92\pm7.91_a$	$30.83\pm8.99_a$	$9.25\pm1.01_b$	$5.46\pm1.08_c$
Butyrate	$44.06\pm3.74_a$	$17.09\pm4.85_b$	$16.12\pm4.44_{bc}$	$29.69\pm2.91_c$	$37.36\pm2.77_{ad}$
Valarate	$14.89\pm1.99_a$	$11.32\pm2.19_a$	$11.73\pm2.44_a$	$11.63\pm3.34_a$	$11.22\pm1.56_a$
Total SCFA	$565\pm20.45_a$	$436\pm23.66_b$	$467\pm23.67_{bc}$	$557\pm13.56_d$	$538\pm17.44_a$

a, b, c, d – the same subscripts in the same row indicate no significant differences ($p>0.05$)

TABLE 4. Ca, Mg and K concentrations in plasma and tibial bone among different study groups.

Group	Control	Furosemide	Vitamin D	FOS	Lactose
Plasma Ca (mg/L)	$84.0\pm0.9_a$	$84.0\pm 4.2_a$	$139.0\pm5.3_b$	$114.2\pm7.1_{bc}$	$89.2\pm4.1_{ac}$
Plasma Mg (mg/L)	$23.7\pm1.8_a$	$26.0\pm4.7_a$	$21.4\pm1.2_a$	$19.2\pm0.8_a$	$19.5\pm3.5_a$
Tibial fresh weight (mg/tibia)	$334.3\pm90.0_a$	$328.3\pm70.2_a$	$332.9\pm20.0_a$	$333.1\pm13.5_a$	$345.0\pm22.3_a$
Tibial dry weight at 105°C (mg/tibia)	$207.3\pm39.4_a$	$201.7\pm74.4_a$	$215.8\pm80.2_a$	$202.4\pm58.6_a$	$209.9\pm80.2_a$
Ash (mg/tibia)	$141.4\pm15.4_a$	$121.4\pm23.2_a$	$121.2\pm11.3_a$	$111.2\pm15.3_a$	$121.3\pm14.4_a$
Ca (mg/tibia)	$45.2\pm0.43_a$	$43.0\pm0.20_b$	$44.6\pm0.36_{ac}$	$45.1\pm0.21_{ac}$	$43.71\pm0.42_{bc}$
Mg (mg/tibia)	$2.12\pm0.67_a$	$1.62\pm0.38_a$	$1.97\pm0.30_a$	$1.84\pm0.16_a$	$2.5\pm0.52_a$
K (mg/tibia)	$2.22\pm0.41_a$	$1.96\pm0.22_a$	$1.84\pm0.38_a$	$1.83\pm0.51_a$	$1.65\pm0.42_a$
Ca/ash (g/100 g)	$33.14\pm0.39_a$	$34.41\pm1.85_a$	$37.24\pm1.98_{ab}$	$40.35\pm0.29_{bc}$	$34.61\pm1.72_{abc}$

a, b, c, same scripts in the same row indicate no significant differences ($p>0.05$)

therapy led to a decrease in bone mineral content. Bushinsky *et al.* [1986] reported hypercalciuria induced by furosemide administration in animals. Fernandez *et al.* [1999] suggested that in patients with renal hypercalciuria there may be an increased release of calcium salts from bone leading to progressive osteopenia. Wong *et al.* [1992] reported hyocalcemia and osteoporosis in an adolescent with renal hypercalciuria, suggesting that, at a high growth rate, sustained renal hypercalciuria may result in negative calcium balance and progressive bone mass loss. Condon et *et al.* [1999] showed that furosemide may decrease calcium levels by 0.5–1 mmol/L in serum but result in a high renal filtered load of calcium which, if above 3.7 mmol/L, causes calcium phosphate protein complexes, thus giving rise to hypercalcaemic nephropathy. The furosemide–treated groups were characterised by increased urinary excretion of Mg and K. Our results are in accordance with findings of Grimm *et al.* [1990] and Atkinson *et al.* [1988] who reported that furosemide treatment increased urinary excretion of Mg and K.

Vitamin D is necessary for normal growth and has complex effects on bone mineralization [Maierhofer *et al.,* 1984]. In hypercalciuric furosemide rats, the data showed the positive effect of $1,25(OH)_2$-vitamin D on calcium deposition into the tibial bone. This effect of $1,25(OH)_2$-vitamin D was likely due to increased calcium absorption and decreased bone turnover [Fernandez, 1999]. In the presence of normal calcium intake, $1,25(OH)_2$-vitamin D has been shown to stimulate intestinal absorption of calcium and phosphorus [Matkovic *et al.,* 1995]. The induced elevation of circulating calcium concentrations, together with a direct action of $1,25(OH)_2$-vitamin D on parathyroid gland receptors, inhibits parathyroid hormone synthesis, giving rise to a decrease in bone turnover [Ordonez *et al.,* 1980]. When dietary calcium is inadequate to maintain blood calcium in the normal range, $1,25(OH)_2$-vitamin D may enhance the mobilization of bone calcium stores, at least when given at pharmacological doses [Maierhofer *et al.,* 1984].

Stimulatory effects of FOS on Ca and Mg absorption were observed in humans and animals [Hirama *et al.,* 2003; Kruger *et al.,* 2003]. FOS increases Ca concentration of the femoral neck [Hirama *et al.,* 2003]. In parallel, the present study indicates that FOS ingestion increased calcium content in tibial bone. Also, FOS increased cecal wall weight and short chain fatty acid production, such as propionate.

Because an adequate supply and a good bioavailability of Ca are essential to attain maximum bone mass on which adult bone status depends, it was important to know the respective effect of FOS on Ca absorption. Ca absorption takes place by two routes. The non-saturable paracellular route of Ca occurs throughout the small intestine and is thought to be driven by passive diffusion. The saturable active transcellular transport is the major absorptive pathway in the proximal intestine (duodenum and jejunum). The large intestine may represent a major site of Ca absorption. In this view, Hylander *et al.* [1980, 1990] showed that the colon plays an important role in the absorption of Ca after small-intestinal resection. In patients with ileostomy, preservation of at least half of the colon improves Ca fractional accumulation in the skeleton. Moreover, rat experiments have shown that fermentable oligosaccharides improve colorectal and cecal absorption of Ca [Demignė *et al.,* 1980; Rėmėsy *et al.,* 1993; Ohta *et al.,* 1995; Hara *et al.,* 1999]. Because FOS is not hydrolysed by enzymes in the small intestine of monogastrics, it reaches the colon intact. FOS is fully metabolised by colonic microflora. End products of carbohydrate fermentation are lactate and SCFA, such as acetate, propionate and butyrate. The present study indicates that FOS ingestion increased SCFA production, such as propionate. The high concentration of organic acids in the cecum leads to a decrease of pH that raises the concentration of soluble Ca. In parallel, in rats fed FOS diets, hypertrophy of the cecum is observed (increase of cecal wall weight) leading to a greater exchange surface area. Thus the enlargement of cecum and the elevation of Ca solubility allow a better cecal absorption of Ca in rats adapted to FOS diets [Lopez *et al.,* 2000]. It is also possible that SCFA can directly stimulate Ca absorption in the rat colon [Luts & Scharrer, 1991] and Ca could pass through the cell membrane more readily in the form of a less charged complex (Ca propionate) by a passive pathway [Trinidad *et al.,* 1993]. Our results are in accordance with findings of Lopez *et al.* [2000] who reported that FOS administration increased bone Ca content. Ohta *et al.* [1995] concluded that FOS feeding completely prevented osteopenia in rats.

In conclusion, vitamin D and oligofructose supplementation led to an increase in Ca content of bone and a decrease in the urinary excretion of Ca, Mg and K in the furosemide--treated rats. Thus, these treatments could be effectively used as a conjunction to furosemide as therapy for raised blood pressure in humans.

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