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Growth Failure and Decreased Bone Mineral of Newborn Rats with Chronic Furosemide Therapy

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ABSTRACT. To test the hypothesis that chronic furosemide treatment in otherwise healthy newborn animals may lead to lowered bone mineral [calcium (Ca) and magnesium (Mg)] content, healthy littermates within each litter of Sprague-Dawley rat pups were randomly assigned to three groups: control, low dose furosemide (5 mg/kg/day), and high dose furosemide (15 mg/kg/day). The pups were treated between days 4 and 28 postnatally. The wet and dry weights of kidneys and tibiae significantly correlated with body weights at sacrifice. Furosemide-treated pups demonstrated a dose-dependent growth delay, decreased total bone (tibiae) Ca and Mg, increased urine Ca and Mg concentration, and a significant inverse correlation between bone Ca and urine Ca concentration. There was no significant difference among the groups when bone Ca and Mg were normalized to per gram of bone dry weight. There were no significant differences among the groups with respect to bone phosphorus or urinary phosphorus concentration; kidney and serum Ca and Mg; or serum sodium, potassium, alkaline phosphatase and immunoreactive parathyroid hormone concentration. We conclude that chronic furosemide therapy leads to growth failure and to increased urinary losses of Ca and Mg. Total bone Ca and Mg in the furosemide-treated pups were diminished in proportion to growth retardation but the bone mineral content per unit of dry weight remained similar to control pups. (*Pediatr Res* 20: 74–78, 1986)

Abbreviations

Ca, calcium
Mg, magnesium
P, phosphorus
Na, sodium
K, potassium
PTH, parathyroid hormone

Potent "loop" diuretics such as furosemide are frequently used in sick newborn infants. Their role in congestive cardiac failure and acute fluid overload is well established (1–3). These diuretics have been proposed to be beneficial for infants with acute (4–6) and chronic respiratory disorders (7). However, not all investigators support these uses (8–10) and there are increasing reports of complications associated with furosemide use in infants (11–15). Anecdotal reports have associated chronic furosemide therapy in human newborns with renal calcification (13) and bone demineralization with secondary hyperparathyroidism (14). These complications are usually described in infants with complicated clinical courses. In these situations, there are usually multiple confounding variables including, *e.g.* variable nutritional intake (or lack of it) that might influence mineral metabolism in the sick infant. The aim of this study is to test the hypothesis that chronic furosemide administration alone can reduce bone mineral content in the healthy newborn rat.

MATERIALS AND METHODS

Healthy newborn littermates of Sprague-Dawley rats were randomly assigned (within each litter by drawing assignments from sealed envelopes) to a control group or to one of two furosemide-treated groups. If the litter size was large, it was culled to a maximum of nine pups to minimize the possibility of reduced or unequal nutritional intake for the pups. A total of nine litters were used.

Various concentrations of furosemide (Lasix, Hoechst-Roussel Ltd., N. Somerville, NJ) were prepared by dilution with preservative free sterile water so that the daily volume of injection remained between 0.1 and 0.4 ml for each pup. The diluted furosemide solutions were kept in a syringe sealed from light at 4° C and fresh solutions were prepared every 48 h. The furosemide treatment included a high dose (15 mg/kg/day) and a low dose (5 mg/kg/day) group. The quantity of furosemide was administered subcutaneously in equally divided doses twice daily from the 4th day until 28 days after birth. The dosage and volume of injection were adjusted twice weekly according to the weight of the pups to the nearest 5 g. The pups were weighed with the same electronic scale (Fisher model 200, Ainsworth Div., Denver, CO) to the nearest 0.1 g. The pups in the control group were given two subcutaneous injections of sterile water of equal volume to the treatment groups. The pups were allowed to nurse *ad libitum* throughout the study.

A spot urine collection was attempted using electrolyte free capillary tubes (Fisher Scientific Co., Cincinnati, OH) applied to the external genitalia of the pups during the morning injection on day 28. Thus the urine was collected 12 h after the previous injection. Six litters were sacrificed on day 28 and three litters were sacrificed on day 31. The pups were sacrificed by exsanguination under ether anesthesia. Serum and urine samples were stored at −20° C until assay.

A standardized radiograph of the whole body was taken of all

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the pups and analyzed for evidence of osteopenia and rickets and for evidence of renal calcification by one radiologist. Right tibia length of each pup was determined from the same radiograph with the same ruler to the nearest mm by one investigator (W.W.K.K.)

The kidneys were then removed and dissected clean of perinephric fat, and as much as possible of the renal pelvis and renal capsule were removed. Shafts of tibiae were dissected free of all soft tissue. Both kidneys were weighed together to the nearest 0.1 mg (Mettler H51AR scale, Mettler Corp., Cleveland, OH) as were both tibiae. Each organ was individually wrapped in parafilm and frozen at −20° C until assay.

Prior to the ashing process, the kidneys and tibiae were placed in a drying oven at 100° C for 10 to 12 h and reweighed at 2- to 3-h intervals thereafter until irreducible dry weight was obtained. The kidneys and bones from each pup were then wet ashed separately in 70% nitric acid at 140° C. The final ash solution was made up to 10 ml with triple deionized water.

The serum, urine, and ashed samples of kidney (both kidneys) and bone (tibiae) were measured for Ca, Mg, and P. In addition, serum concentrations of Na, K, alkaline phosphatase, and immunoreactive PTH were measured.

Ca and Mg concentrations of the serum and the ash sample were measured by atomic absorption spectrophotometry (16). P concentrations were measured by the method of Fiske and Subbarrow (17). Serum concentrations of Na and K were measured by ion selective electrode (IL 508, Lexington, MA); alkaline phosphatase was measured by the method of Wilkinson et al using a centrifugal analyzer (18). iPTH was measured by PTH-midmolecule radioimmunoassay (Immuno Nuclear Corp., Stillwater, MN) with the addition of a rat standard (1000 pmol/liter). Units are expressed as picomoles human PTH equivalent (hPTH-eq)/liter. The antiserum measures the midmolecule (44–68) amino acid sequence of the PTH molecule (19). In our laboratory, the interassay coefficient of variation for this assay is 9.6%.

Analysis of covariance (20) was used to assess the effects of furosemide treatment and postnatal age on body weight for each group. One-way analysis of variance and Student Newman Keuls multiple range test were used to determine the differences among the three groups. Standard linear regression analysis were performed as indicated. Statistical calculations employed the CLINFO system software and were supplemented by the SAS program (21) of the NIH General Clinical Research Center, University of Cincinnati.

RESULTS

Twenty-five pups were randomly assigned to the control group, 25 to the low dose furosemide (5 mg/kg/day) group and 27 pups to the high dose (15 mg/kg/day) furosemide group. On day 4 after birth, at the beginning of the study, there were no differences in the body weight of the pups among groups. There was a dose

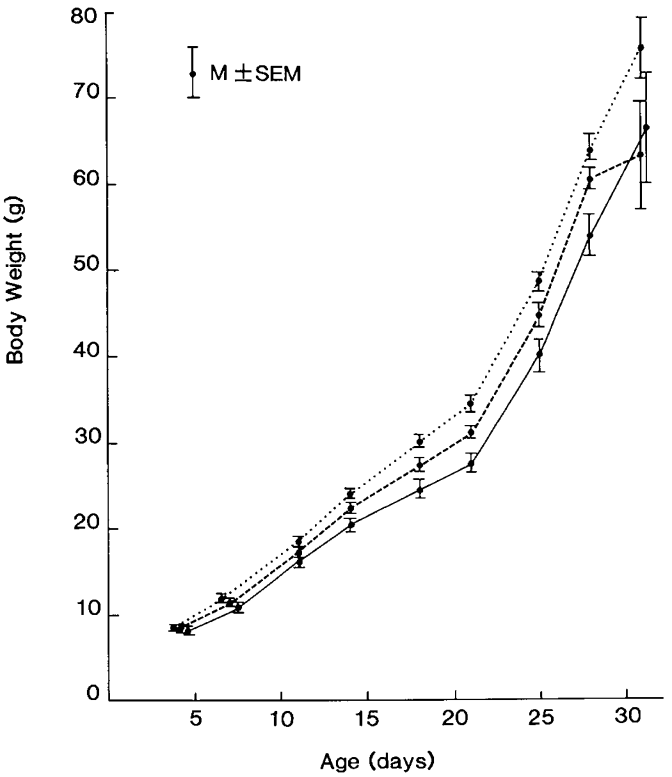

Fig. 1. Body weight (mean ± SEM) of rat pups in the control and furosemide treated groups. ●●●●, control group ($n = 25$); - - -, low dose furosemide 5 mg/kg/day group ($n = 25$); ----, high dose furosemide 15 mg/kg/day group ($n = 27$). Analysis of covariance for postnatal age and body weight. $p < 0.004$ low dose furosemide group compared to control group; $p < 0.001$ high dose furosemide group compared to control group; $p < 0.03$ high dose furosemide group compared to low dose furosemide group.

Table 1. *Changes in body wt, bone* and kidney* wt with various treatment regimens (mean ± sem)*

		At sacrifice		Bone		Kidney	
Group	*n*	Body wt (g)	Right tibia length (mm)	Wet wt (mg)	Dry wt (mg)	Wet wt (mg)	Dry wt (mg)
Control	25	68.1 ± 1.8	24.0 ± 0.2	210.4 ± 8.4	125.4 ± 4.3	598.6 ± 20.1	154.0 ± 4.4
r†				0.56	0.54	0.78	0.68
p				0.003	0.006	<0.001	<0.001
Furosemide, 5 mg/kg/day	25	62.1 ± 2.1 (91.5 ± 3.1)§	23.1 ± 0.2 (96.4 ± 1.0)	172.7 ± 7.9‡ (82.6 ± 3.8)	109.7 ± 4.2‡ (82.6 ± 3.0)	535.1 ± 21.7 (89.9 ± 3.1)	130.6 ± 5.9‡ (85.1 ± 3.6)
r				0.63	0.77	0.81	0.46
p				<0.001	<0.001	<0.001	0.02
Furosemide, 15 mg/kg/day	27	57.3 ± 3.2‡ (84.3 ± 4.6)	22.7 ± 0.4‡ (94.6 ± 1.9)	158.5 ± 9.9‡ (75.9 ± 4.8)	101.7 ± 5.1‡ (81.0 ± 4.1)	505.1 ± 30.6‡ (84.9 ± 4.9)	127.2 ± 7.5‡ (83.1 ± 4.9)
r				0.86	0.93	0.93	0.79
p				<0.001	<0.001	<0.001	<0.001

* Bone wt includes both tibae; kidney wt includes both kidneys from same pup.

† Correlation with body wt at sacrifice, linear regression analysis.

‡ $p < 0.05$, compared to control group; one-way analysis of variance and Newman Keuls multiple range test.

§ Numbers in parentheses are percentage of mean wt of littermate controls.

dependent growth delay in the furosemide treated groups. The effect of age on weight was significantly different in each group, *i.e.* the shape of the growth curve for each group (best approximated by including a quadratic term for age) was significantly different from the other groups (Fig. 1).

There was significant lowering of body weight (Fig 1), of right tibia length, and of wet and dry weight of bone and kidney (Table 1) in the furosemide treated groups compared with the control group. There were no significance differences in the percent decrease in body weight and right tibia length at sacrifice for each of the treated groups.

Both the wet weight and dry weight of bone and kidney were correlated with body weight at sacrifice (Table 1). There were significant decreases in total bone (tibial) Ca and Mg in the furosemide treated groups compared with the control group. There were no differences among the groups with respect to total bone P and kidney Ca, Mg, and P contents (Table 2). There were no differences among the groups if the bone and kidney Ca, Mg, and P were normalized to per gram dry weight of tissue (Table 2).

There were no differences among the groups for any of the serum variables measured (Table 3). Attempts at urine collection were made on days 13, 14, 27, and 28 days, but these attempts were successful only on day 28. Urinary Ca and Mg concentrations on day 28 were significantly increased in the high dose furosemide group while urinary P concentrations were variable and did not show any difference among the groups (Table 4). Bone (tibial) Ca concentration was inversely correlated with urine Ca concentration (Fig. 2).

Whole body radiographs of the pups did not demonstrate any discernible bone abnormalities or renal calcification.

DISCUSSION

In this study, chronic furosemide therapy is associated with significant growth retardation and reduced total bone (tibial) Ca and Mg in young rat pups. Although the total bone P was not significantly reduced, it was reduced proportional to the Ca as would be expected. However, bone Ca, Mg, and P, when expressed per gram of bone dry weight, were not different between the control and treated pups. These findings would imply that furosemide treatment results in a growth-retarded pup with small but normally mineralized bone. Biochemical and hormonal homeostasis as indicated by normal serum concentrations of Ca, Mg, P, and Ca regulating hormone would further support the likelihood of small but normal bones in furosemide-treated pups. Similarity in bone radiographs also may be due to similar bone Ca, Mg, and P concentrations among the groups. Decreased bone mineral in this study is probably related to at least two mechanisms—growth delay and increased urinary Ca and Mg loss.

The growth delaying effect of chronic furosemide treatment may vary with the age at which therapy was begun. Warshaw *et al.* (22) demonstrated that furosemide at a dose of up to 200 mg/kg/day when administered to rats with food for 25 days resulted

Table 4. *Urine biochemical measurements of rat pup urine on last day of furosemide treatment (day 28) (mean ± sem)*

Group	Ca (mg/dl)	Mg (mg/dl)	P (mg/dl)
Control	8.4 ± 0.9 (4)*	11.9 ± 2.2 (4)	82.5 ± 26.6 (4)
Furosemide, 5 mg/kg/day	11.3 ± 1.6 (9)	14.1 ± 1.4 (9)	80.4 ± 17.3 (6)
Furosemide, 15 mg/kg/day	20.3 ± 3.5† (8)	19.3 ± 1.4† (8)	79.0 ± 9.4 (5)

* Numbers in parentheses are number of pups measured.

† $p < 0.05$ when compared to the control group. One-way analysis of variance and Newman Keuls multiple range test.

Table 2. *Ca, Mg, and P content of bone (both tibae) and both kidneys (mean ± sem)*

	Bone			Kidney		
Group	Ca (mg)	Mg (mg)	P (mg)	Ca (mg)	Mg (mg)	P (mg)
Control ($n = 25$)	19.77 ± 0.70 (157.7 ± 1.91)*	0.49 ± 0.02 (3.87 ± 0.07)	9.43 ± 0.47 (74.9 ± 2.41)	0.76 ± 0.07 (4.96 ± 0.37)	0.14 ± 0.01 (0.93 ± 0.03)	1.43 ± 0.06 (9.32 ± 0.35)
Furosemide, 5 mg/kg/day ($n = 25$)	17.47 ± 0.71† (158.8 ± 1.66)	0.43 ± 0.01† (3.98 ± 0.08)	8.31 ± 0.41 (76.2 ± 3.03)	0.71 ± 0.05 (5.72 ± 0.47)	0.13 ± 0.01 (1.08 ± 0.10)	1.32 ± 0.06 (10.59 ± 0.68)
Furosemide, 15 mg/kg/day ($n = 27$)	16.44 ± 0.82† (162.1 ± 1.54)	0.42 ± 0.02† (4.31 ± 0.28)	8.08 ± 0.40 (78.4 ± 2.13)	0.64 ± 0.05 (5.28 ± 0.33)	0.13 ± 0.01 (0.99 ± 0.07)	1.37 ± 0.08 (11.09 ± 0.58)

* Numbers in parentheses are Ca, Mg, and P content/g dry wt of bone and kidney, respectively.

† $p < 0.05$, compared to control group; one-way analysis of variance and Newman Keuls multiple range test.

Table 3. *Serum biochemical and hormonal measurements in rat pups (mean ± sem)*

	Serum						
Group	Ca (mg/dl)	Mg (mg/dl)	P (mg/dl)	Sodium (mEq/liter)	K (meq/l)	Alkaline phosphatase (IU/liter at 30° C)	Immunoreactive parathyroid hormone pmol (human PTH-eQ)/liter)
Control	10.5 ± 0.2 (25)*	2.97 ± 0.07 (25)	11.6 ± 0.4 (25)	144 ± 0.6 (21)	6.0 ± 0.2 (21)	412 ± 22 (25)	170 ± 8 (24)
Furosemide, 5 mg/kg/day	10.2 ± 0.2 (24)	2.85 ± 0.07 (24)	11.6 ± 0.3 (24)	146 ± 0.6 (20)	6.2 ± 0.2 (20)	412 ± 26 (24)	170 ± 6 (20)
Furosemide, 15 mg/kg/day	10.7 ± 0.3 (24)	3.05 ± 0.08 (24)	11.6 ± 0.3 (24)	146 ± 1.2 (19)	5.8 ± 0.3 (17)	433 ± 21 (23)	161 ± 11 (20)

* Numbers in parentheses are number of rat pups measured.

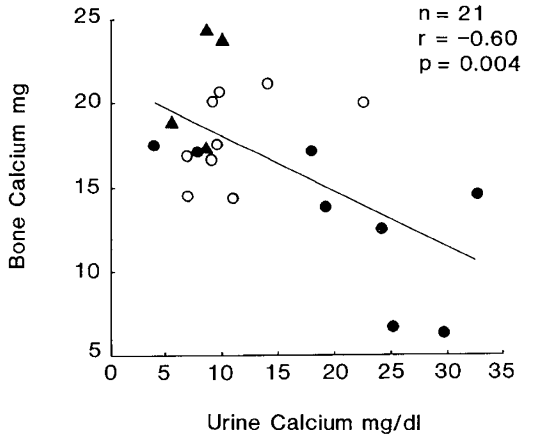

Fig. 2. Correlation between bone (tibial) calcium content and urine calcium concentration. *Solid triangle*, control group; *open circle*, low dose furosemide (5 mg/kg/day) group; *closed circle*, high dose furosemide (15 mg/kg/day) group.

in an approximately 4.6% lower body weight compared to controls. In contrast, in this study of newborn rats, we demonstrated that the onset of growth delay in the furosemide-treated pups was early and marked. The extent of growth delay was proportional to the dose of furosemide given to the pups. During the study period, the increase in the mean body weight of control pups was approximately 9-fold compared to an approximately 7-fold increase in the mean body weight in the furosemide-treated groups. At the time of sacrifice, the difference in mean body weight between furosemide-treated and control pups was up to 16.5%. It might be that younger animals in the present study, compared with 6-wk-old animals in the earlier study (22), are more sensitive to the growth delaying effect of furosemide treatment.

The mechanism of growth delay in chronic furosemide treatment is not known. In the rat, there is no agreement on whether growth delay is secondary to the effect of furosemide on Na depletion (22, 23). In humans, a renal compensatory effect of reduced Na excretion with chronic furosemide treatment has been reported (24). In the present study, the lowered body weight of the furosemide-treated pups probably was not due to extracellular or total body fluid changes, since there were no differences in serum concentrations of Na, K, Ca, Mg, and P among the groups, and organ (bone and kidney) wet and dry weights were highly correlated with body weight on sacrifice.

Diminished weight gain associated with metabolic alkalosis secondary to chloride deficiency has been reported in infants (25) and in growing rats (26, 27). This may be related to the loss of appetite described in infants (25), but the effect on food consumption in growing rats may be variable (22, 26, 27). Chronic furosemide administration also can induce a state of hypochloremic metabolic alkalosis (28). However, the normal serum K concentration of the pups in this study would offer indirect evidence against the presence of metabolic alkalosis. The use of littermates as control and treated groups, and, by design, limitations of litter size, should have minimized differences in nutrient intake for the pups.

Theoretically, chronic furosemide treatment may lead to growth delay by a number of other effects, *e.g.* altered body mineral (K, chloride) and trace element (zinc) status (28, 29–32). Regardless of the mechanism(s) involved in growth delay, the slowing in the rate of weight gain became less pronounced with age, indicating possibly the ability of rat pups to at least partially compensate for the growth delaying effect of furosemide treatment.

It is well known that furosemide can increase urinary Ca excretion in human adults (33, 34), infants (13), and animals (22); the urinary excretion of Mg also is increased after furosemide administration (22, 28). From our data, it would seem that the urinary losses of Ca and Mg did not result in lowered serum concentrations of Ca and Mg and thus did not stimulate PTH release. In this study furosemide treatment was not associated with elevation in serum PTH concentrations. Since serum alkaline phosphatase concentrations were similar in all groups, increased bone turnover secondary to PTH excess also would be unlikely. Variable urinary P excretion has been reported in other studies of furosemide administration (13, 28). In this study, the effect of furosemide treatment on urinary P loss was variable and not increased. The lack of increased urinary P also would support the absence of secondary hyperparathyroidism in chronic furosemide treatment.

In hypercalciuric states (35, 36), it is conceivable that an increased gastrointestinal absorption of Ca may compensate for the urinary losses. However, this compensatory response is only partially effective; chronic furosemide therapy in 6-wk-old Sprague-Dawley rats has been associated with less positive Ca balance when compared to controls (22). From our data, it appears that intestinal Ca absorption was appropriate to meet the lowered demands of the slower growth rate of the furosemide-treated pups. Similar mechanisms might be operative for Mg metabolism, although there is no information in that regard. Nevertheless, it is conceivable that bone mineralization may be adversely affected to a greater extent if the balance among mineral needs for growth (which in this instance is reduced), mineral supply (mineral nutrient content and intestinal absorption), and mineral loss (from the prolonged use of potent diuretic) is disturbed. Thus, in theory, chronic furosemide therapy may be more likely to result in decreased bone mineralization in preterm infants who are fed human milk with mineral content that is low in relation to theoretic high mineral needs (37), when compared to rat pups which were fed rat milk with high mineral content appropriate for mineral needs (38). The inverse correlation between the urine Ca concentration and total tibial Ca content may be an additional factor associated with lowered bone Ca.

In the present study there was no accumulation of Ca and Mg in the kidney tissue of furosemide-treated pups. Although the mean urinary Ca and Mg concentrations of the treated pups were approximately doubled compared to control pups, it is possible that the extent of urinary loss of Ca and Mg may be greater than that indicated from our results, since, because of technical reasons, urine samples were obtained only 12 h after the previous injection of furosemide. In infants, the peak urine Ca and Mg concentration from furosemide therapy is likely to be much greater than that measured in this study; urine excretion of Ca in infants who received furosemide therapy has been reported to be 10-fold higher than that of controls (13). Thus even in the absence of other factors such as acid base disturbances associated with chronic lung disease which may precipitate or aggravate any tendency to renal calcium precipitation, the prolonged use of potent diuretic theoretically can still predispose the infant to renal calculi and nephrocalcinosis (13).

In the present study, we demonstrated that in the young rat model, prolonged use of the potent diuretic furosemide has a growth inhibitory effect. Although the decreased total bone mineral is proportional to the growth delay, *i.e.* the bone mineral concentration remained normal, it is conceivable that in situations where a diminished supply or absorption of minerals occurs, such as frequently occurs in sick human preterm infants, there may be greater disturbances to bone mineralization as a result of chronic diuretic therapy.

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