Development of an animal model of nephrocalcinosis via selective dietary sodium and chloride depletion

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Abstract

Background—Nephrocalcinosis (NC) is an important clinical problem seen in critically ill preterm neonates treated with loop diuretics. No reliable animal models are available to study the pathogenesis of NC in preterm infants. The purpose of this study was to develop a reproducible and clinically relevant animal model of NC for these patients, and to explore the impact of extracellular fluid (ECF) volume contraction induced by sodium and chloride depletion in this process.

Methods—Three-week old weanling Sprague-Dawley rats were fed diets deficient in either chloride or sodium and chloride. A sub-group of rats from each dietary group was injected daily with furosemide (40 mg/kg; i.p.).

Results—Rats fed a control diet, with or without furosemide, or a chloride depleted diet alone, did not develop NC. In contrast, 50% of the rats injected with furosemide and fed the chloride depleted diet developed NC. Moreover, 94% of the rats fed the combined sodium/chloride depleted diet developed NC, independently of furosemide use. NC was associated with the development of severe ECF volume contraction, hypochloremic, hypokalemic metabolic alkalosis, increased phosphaturia, and growth retardation.

Conclusion—Severe ECF volume contraction induced by chronic sodium and chloride depletion appears to play an important role in the pathogenesis of NC.
INTRODUCTION

Nephrocalcinosis (NC) is a well-described complication seen frequently in pre-term infants and in older children with inherited and acquired renal tubulopathies. Furthermore, NC is a well described complication of congenital chloride diarrhea (CLD), which with time, contributes to the renal injury seen in these patients (1). The pathogenesis of NC in pre-term neonates has been associated with the use of chronic loop diuretics, as well as the presence of electrolyte changes or critical illness (2, 3). However, the basic pathogenic mechanisms involved in NC are not clearly understood, and there has been no definitive link between the development of NC in pre-term neonates and the duration or dose of loop diuretic therapy. Estimates of the incidence of NC based on screening of pre-term infants range from 7% to 39% (4–7). NC has been associated with the development of renal insufficiency and hypertension in both pre-term neonates treated with furosemide, in infants with inherited renal tubulopathies, and in children with CLD (8–11). Hypercalciuria (HC) has not been a necessarily consistent finding in all infants with NC (12, 13) and is also not typically present in children with CLD (8) which speaks to the non-essential role of HC in the pathogenesis of this disease. NC does not uniformly resolve in infants who are taken off loop diuretic therapy and certainly persists in children with inherited tubulopathies and CLD (9, 14, 15). Furthermore, the ability of loop diuretics to promote NC in preterm infants does not seem to result from an increase in stone promoting substances such as oxalate (16).

Currently, no animal models exist that attempt to replicate the fluid and electrolyte abnormalities that are associated with the development of NC in the pre-term infants. A prior model using i.p. furosemide alone in weanling rats on a control diet was able to induce NC (17), however these changes could not be reproduced in our preliminary experiments. Also, furosemide has not been shown to induce NC in adult rats fed control diets (18). Other studies in adult rats have produced NC using a combination of chloride depletion with either phosphate loading or high sulfate diet (18, 19). Isolated phosphate loading has consistently produced NC only in young rats (20). Therefore, the purpose of the current study was to develop a reliable and reproducible animal model system of NC that mimics the pathophysiologic alterations associated with the development of NC in pre-term neonates receiving furosemide, children with inherited renal tubulopathies, or children with extra-renal losses of volume and electrolytes such as occurs in CLD or pyloric stenosis.

RESULTS

Chloride and sodium deficient diets induced NC in young rats

The most remarkable finding was that almost all rats fed the combined sodium and chloride deficient diet, developed moderate to severe NC (p-value < 0.001 when compared to controls) (Table 1). Representative histologic samples from these groups and controls are shown in Figure 1. Young rats treated with furosemide alone, or fed a selective chloride deficient diet alone, developed hypokalemic, hypochloremic, metabolic alkalosis, but, these changes were not sufficient to induce NC (Table 1). However, 50% of the chloride depleted rats treated with i.p. furosemide developed moderate to severe NC (p-value < 0.01). These changes were also associated with the development of hyponatremia and increased urinary sodium excretion (Table 2). In general, animals that developed more severe metabolic
alkalosis (e.g. higher serum bicarbonate concentration) also showed lower levels of ionized calcium.

Examination of von Kossa and H&E (not pictured) stained rat kidney sections under polarized light failed to reveal birefringent crystals in animals with NC. This, as also confirmed on Alizaren red stains (Figure 1), show that most deposits were composed primarily of calcium-phosphate. None of rats fed a control diet developed NC.

**NC induced through severe ECF volume contraction**

Rats that developed severe ECF volume contraction, as evidenced by decreased weight, increased hematocrit, and increased blood urea nitrogen were more likely to develop NC (Table 3). However, selective chloride deficient rats developed significant ECF volume contraction but did not develop NC. These findings suggest the ECF volume contraction per se, is not sufficient to induce NC. Rats fed a low sodium/chloride diet had a higher mean urine output (ml/day) (p<0.05) than rats fed a control diet (Table 3). In the context of ECF volume contraction, this represents inappropriate urinary fluid losses. Rats who were administered i.p furosemide with either a low chloride or low sodium and chloride diet did not have a higher daily urine output than rats who were given the respective diets alone (Table 3).

**Urine calcium excretion in rats with NC**

As shown in Figure 2, rats from dietary intervention groups (e.g. low chloride diet + furosemide or low sodium and chloride diet alone) that developed NC had mean urine calcium to creatinine ratios that were not different than animals fed a control diet. Across all dietary intervention groups, rats that developed NC had a significantly lower urine calcium to creatinine excretion (0.22 vs. 0.31 mg/mg creatinine, p < 0.05) than those that did not. This relationship also held true within the group of rats fed a low chloride diet and given i.p. furosemide in which 50% of animals developed NC. Among this group, urine calcium excretion was significantly lower in rats that developed NC (0.18 vs. 0.29 mg/mg creatinine, p < 0.05). Across all study groups, the ionized calcium (mean +/- SD) was 1.13 +/- 0.11 among rats with NC and 1.26 +/- 0.12 in rats without NC (p < 0.001). A decreased whole-blood ionized calcium level was associated with a hypochloremic, hypokalemic, metabolic alkalosis in all rats (Table 2). In a logistic regression model examining urinary parameters associated with NC, a lower urinary potassium excretion (β-coefficient (β)=-3.8, p < 0.01) and a higher 24-hour urine output (β=0.02, p=0.05) were also associated with the development of NC.

**Association of urine phosphorus excretion with NC**

As shown in Table 2, urinary phosphorus excretion was over two-fold higher among rats fed a low sodium and chloride diet compared with controls. Also, among rats fed a low chloride diet who were administered i.p furosemide, those that did develop NC had a 10-fold higher urinary phosphorus excretion than those that did not (p < 0.05). The lowest urine phosphorus excretion was seen among rats fed a low chloride diet alone speaking against increased urine phosphorus excretion resulting directly from chloride depletion. Urine magnesium excretion did not show a definitive association with NC despite rats fed a control diet having the
The highest magnesium excretion occurred among rats fed a low chloride diet alone which did not induce NC in the absence of concurrent i.p. furosemide administration.

Effects of intraperitoneal furosemide administration in dietary groups

As shown in Table 2, among rats fed the chloride deficient diet, i.p. furosemide administration caused a further drop in serum sodium (p < 0.05), serum potassium (p < 0.01), and serum chloride (p < 0.001). This same pattern held true, as expected, for rats fed control diets. In the case of rats fed the control diet, the drop in serum sodium and chloride could be attributed in part to furosemide’s effect on increased urinary excretion of sodium (p < 0.001) and chloride (p < 0.001). However, among rats fed the chloride deficient diet, there was no significant difference in daily urinary sodium, potassium, nor chloride excretion in animals injected with i.p. furosemide. Also, i.p. furosemide administration was associated with an increased arterial pH due to metabolic alkalosis in rats fed the control diet (p < 0.05) but not among rats fed the chloride deficient diet who had pre-existing alkalosis due to hypochloremia with ECF volume contraction.

Blood pressure among rats with and without NC

An important negative finding of this study was that there was no difference in systolic, diastolic, nor mean blood pressure among rats that did and did not develop NC. As stated above, rats that developed NC had more severe ECF volume contraction when compared to those that did not develop NC. This however was neither associated with an increased nor decreased blood pressure in these animals.

Growth in rats fed chloride or sodium/chloride deficient diets

As shown in Figure 3, growth (e.g. weight gain) significantly differed among dietary intervention groups (p<0.001) with the chloride deficient rats having the poorest weight gain followed by rats fed the low sodium/chloride deficient diet in comparison to controls. Rats fed a control diet, had normal weight gain. Among rats fed a low chloride or low sodium/chloride diet, those injected with furosemide had poorer weight gain than non-injected animals (p-value <0.05). There was no difference in weight gain among rats fed a control diet in the presence or absence of furosemide treatment. At the time of euthanasia (e.g. 7 weeks of age) there was a significant difference in length (13.4 cm vs. 20.1 cm, p <0.001) in rats fed a low chloride versus control diet. There was no difference in length between rats fed a low chloride diet and those receiving a sodium/chloride depleted diet. Rats from the low chloride + furosemide and low sodium and chloride dietary groups had lower absolute daily voluntary food intake relative to controls (Table 3). There was no difference in daily food intake between rats in the low chloride + furosemide and low sodium and chloride dietary groups.

DISCUSSION

The treatment of pre-term neonates with furosemide has been associated with the development of NC and renal injury. The mechanisms by which furosemide induced these changes are not clearly understood, and there is a need to develop a reliable small animal model...
To address this issue, we developed a rat model of NC, and explored the role of furosemide, sodium, and chloride depletion in the pathogenesis of NC. Surprisingly, we found that severe chronic dietary sodium and chloride depletion consistently induced NC in young Sprague-Dawley rats not treated with furosemide. These findings were associated with severe extracellular fluid volume contraction, hypochloremic, hypokalemic metabolic alkalosis, and increased urine phosphorus excretion (Table 1). Of interest, we did not detect NC in young rats treated with furosemide and fed a control diet. In contrast, 50% of the rats treated with furosemide while fed a selective chloride deficient diet, developed NC. Taken together, these findings suggest that the three major trigger mechanisms by which furosemide may precipitate the development of NC in pre-term infants are through the induction of ECF volume contraction with hypochloremia, increased urine phosphorus excretion, and hypercalciuria. However the potential role of hypercalciuria could not be confirmed in our animal model system.

There are several potential clinically relevant findings that can be derived from these data. First, this study describes a reliable small animal model system to study the pathogenesis of NC in pre-term infants. This model produces reliable and severe NC in a significant proportion of rats (50% – 100%) (Table 1). Previous studies have shown that weanling rats provide a clinically relevant model system to study how chronic changes in dietary sodium and chloride depletion modulate the ECF fluid volume, total body growth, muscle protein synthesis, and the activation of the systemic and local renin-angiotensin system (21–24). The rapidly growing organism of preterm infants and young rats, needs electrolytes to support the expansion of ECF volume required for normal growth (21, 25–27). Furthermore, young rats are more susceptible to develop NC when compared to adult rats (28) and they are more sensitive to the effects of dietary sodium/chloride restriction (26). In agreement with previous studies (21, 25), our data, suggest that the stunted growth seen in rats fed the sodium and/or chloride deficient diets, can be at least partially explained by the reduced caloric intake and severe ECF volume contraction.

Second, our model speaks to the importance of both sodium and chloride depletion in addition to volume contraction in the pathogenesis of NC. For example, sodium supplementation has been shown to improve growth and prevent NC in Sprague-Dawley rats who were treated with i.p. furosemide for four weeks (27). Furthermore, as previous clinical data also suggest, calcifications do not resolve in all animals after discontinuation of furosemide treatment (17). However, in the present and previous studies we found that rats fed selective sodium deficient diet did not develop NC (21). Our findings provide strong support to the notion that a combination of severe dietary sodium and chloride depletion leading to significant ECF volume contraction may play a key role in the pathogenesis of NC in pre-term neonates. For reasons of optimization of lung function, these patients may be subjected to fluid restriction and/or furosemide administration leading to ECF volume contraction. Hypochloremic metabolic alkalosis is associated with bicarbonaturia and increased urinary sodium and potassium losses. Thus, chronic chloride depleted rats also become sodium and potassium depleted. The impact of sodium chloride and volume depletion in the pathogenesis of NC have been demonstrated in children with congenital chloride diarrhea (15). Nevertheless, it should be noted that the rats fed the selective chloride deficient diet alone, showed severe ECF volume contraction and hypochloremic
hypokalemic metabolic alkalosis, but did not develop NC. These findings argue very strongly in favor of the hypothesis that other risk factors (e.g. increased urine phosphorus excretion, hypercalciuria), in addition to ECF volume contraction and hypochloremia, are needed to develop NC. Unfortunately, we were unable to generate a definitive conclusion regarding the potential role of hypercalciuria in our rat model, since furosemide did not appear to induce significant hypercalciuria in chloride depleted rats who developed NC (Figure 2). A similar finding was detected in previous studies done in chloride depleted adult rats (18). In contrast, increased urine phosphorus excretion appears to be a constant finding in young rats with NC (Table 2). For example, the rats fed the combined sodium and chloride restricted diet, had the highest proportion (nearly 100%) and severity of NC as well as the highest phosphorus excretion of all intervention groups (Table 2). Interestingly, weanling rats fed the selective chloride depleted diet showed very low values of urinary phosphorus, when compared to all other groups. These findings may be explained due to the lower phosphorus content of the chloride deficient diet (31% of the control diet), decreased food intake seen in the rats fed a low chloride diet who were given furosemide, and the severe ECF volume contraction changes found in these rats, which should further increase the reabsorption of phosphorus in renal proximal tubules. Nevertheless, when assessing the results in the selective chloride deficient rats treated with i.p. furosemide, we noted that the 50% of the rats who developed NC had a 10-fold higher urinary phosphorus excretion compared to those who did not develop disease. Prior animal studies have stressed the importance of phosphorus and sulfate loading in the development of NC with chloride restriction (18, 19). Thus, it is tempting to speculate that the low urinary phosphorus values detected in selective chloride deficient rats may play a protective role to prevent NC. Alternatively, we did not exclude the possibility that increased phosphaturia detected in all rats who developed NC, may be a secondary event, rather than the primary pathogenic factor for NC. In either case, more studies are needed to clarify these important issues.

Previous studies in humans have not consistently revealed a phosphaturic effect of furosemide (29) and the effect in animals has varied depending on the presence of parathyroid hormone (30). Although NC in our young rats was associated with higher urinary excretion of phosphorus, it is clear that this was not due to the direct effects of furosemide, as the highest phosphate excretion occurred in the rats fed with the combined chloride and sodium deficient diet who were not treated with furosemide (Table 2). Given that the composition of the calcium aggregates seen on histology were in large part composed of calcium-phosphate (Figure 1) and the fact that increased phosphaturia seems to be highly associated with the development of NC in our rat model (Table 3), changes in parathyroid hormone (PTH) secretion or sensitivity may have an important role in the process as well. Indeed, as shown in Table 2, the 2 groups with the highest serum bicarbonate concentration had a more significant decrease in serum ionized calcium concentration, which could have stimulated PTH secretion. Furthermore, a previous study showed that PTH serum levels were increased in rats treated with furosemide that developed NC, and that the severity of NC and kidney calcium content was reduced in rats treated with the calcimimetic NPS R-46731 (31). It is worth mentioning that in our study, unlike prior studies (17), we failed to induce NC by injecting i.p. furosemide in young rats subjected to a control diet. The reason for this is not currently understood, but other groups also failed to
induce NC in rats injected with furosemide alone (18). Thus, factors, in addition to furosemide and PTH, should be considered. In any case, this is an important area for future research endeavors.

Studies on NC developing in the context of a high phosphorus diet or magnesium depletion have not shown regression when animals were switched to control diets (32, 33). Whether this is also the case in our animal model is an area requiring further investigation. It is unclear whether NC associated with sodium/chloride depletion is associated with long-term alterations in renal function. NC induced by excessive dietary phosphorus has been associated with increased urine albumin excretion (34).

There were a few limitations associated with our study. The first is that kidney calcium content was not measured directly. However, prior studies have shown that kidney calcium content strongly correlates with the severity of NC (35). The second is the lack of longitudinal time course follow-up to determine the minimum amount of time required to develop NC as well as the initial changes in urinary phosphorus and electrolytes excretion. The third is that without measurement of PTH in our animal model, obtaining a comprehensive understanding of the pathogenesis of NC is limited. Fourth, we could not directly determine the calciuric effect of furosemide within each study group as paired urine specimens were not obtained pre- and post-furosemide administration within each rat. Finally, we used serum creatinine, blood urea nitrogen, and hematocrit levels to estimate renal function and ECF volume status. Nevertheless, in previous studies using a similar rat model system, we showed that in the absence of bleeding disorders, these markers can be correlated with changes in the renin-angiotensin-aldosterone system and ECF volume contraction (22).

In summary, we have developed a reliable rat model system to study the pathogenesis of NC in pre-term infants. We conclude that chronic dietary sodium and chloride depletion leading to severe ECF volume contraction, hypochloremic metabolic alkalosis, and increased phosphaturia, constitutes a major risk factor for the development of NC in young rats. These findings, suggest that furosemide, may precipitate the development of NC in pre-term infants, at least partially through this simple mechanism. Further studies are needed to validate this hypothesis in pre-term infants, and to elucidate the basic mechanism involved in the pathogenesis of NC in this new animal model system.

**METHODS**

The following protocol was approved by the Children’s National Medical Center Research Institute Institutional Animal Care and Use Committee.

**Subjects and protocol**

Three week old, male Sprague-Dawley rats (Taconic - Germantown, NY) were fed diets deficient in sodium (<0.02%), chloride (<0.005%), or both electrolytes for four weeks (n=12–17 rats per group). Control rats were fed a standard rodent diet (0.13% sodium, 0.54% potassium, 0.55% calcium, 0.56% phosphorus, and 0.03% sulfate). The sodium and combined sodium/chloride deficient diets contained identical amounts of calcium,
phosphorus, and sulfate relative to the control diet. Compared to the control diet, the selective chloride deficient diet had 0.46%, 0.31% and 0.55% of the calcium, phosphorus, and sulfate content respectively. The caloric content of all diets was approximately 4 kcal/g (3.86 to 4.1 kcal/g) with the control, low sodium, and low sodium/chloride diet having slightly more calories obtained from protein (Casein 20%) than the low chloride diet alone (Casein 18.3%). All diets were provided thru MP Biomedicals (Solon, OH). Deionized water was administered to all animals. A sub-group of rats from each dietary intervention group was administered 40 mg/kg of furosemide via daily i.p. injection. Metabolic cages were used to collect 24-hour urine specimens and measure daily food intake in sub-groups of rats between six and seven weeks of age. Animals were euthanized at 7 weeks of age. At the time of euthanasia, animals were anesthetized with either pentobarbital (35 mg/kg) or ketamine (80–100 mg/kg and xylazine (5–10 mg/kg).

**Measurements**

Urine calcium was measured via colorimetric assay (mg/dl, inter-assay coefficient of variation (CV) = 0.9%). Urine creatinine was measured via photoabsorbiometry utilizing the modified Jaffe reaction (mg/dl, CV=1.1%). Urine sodium (mmol/l, CV= 3.8%), potassium (mmol/l, CV=2.6%), and chloride (mmol/l, CV=2.4%) were measured via ion selective electrodes. Daily water intake was measured in all animals while in metabolic cages. Just prior to euthanasia, blood pressure and heart rate was measured via indwelling arterial transducer (femoral or carotid access). Whole blood was collected in lithium heparin tubes via arterial catheter for electrolytes (sodium, potassium, chloride), ionized calcium, pH, and bicarbonate. Hematocrit was sampled via arterial line and measured via a hemocytometer. Kidneys were harvested from all animals and sectioned longitudinally, fixed in 10% buffered formalin, and stained with both von Kossa (non-specific calcium) and Alizarin red (calcium-phosphate specific) stains. All renal histology slides were reviewed by study investigators for the presence of calcium deposition in the renal parenchyma. A subset of slides with confirmed NC via von-kossa were H&E stained and viewed under polarized light for the presence of calcium oxalate aggregates. NC severity was graded on a 1 to 5 scale by study investigators based on the proportion of renal tubular parenchyma with calcium deposition on von Kossa staining. Severe NC was defined as grades 4 to 5.

**Data analysis**

A 24-hour urinary for calcium excretion was collected after 4 weeks of exposure to each diet and analyzed as mg calcium per mg creatinine (mg/mg). 24-hour urine output was analyzed as ml/day and daily fluid intake was converted to ml/100g/day for all analyses. Food intake was analyzed as g/day. All statistical analyses were performed on STATA 11. Results of weight gain, fluid intake, as well as urine and blood measures were expressed as means plus or minus (+/−) standard deviations (SD) for each dietary group. One way analysis-of-variance (ANOVA) was used to evaluate differences in serum and urine electrolytes among the eight different groups of rats and the students’ t-test to make comparisons between groups. Urine and blood parameters were compared between rats that did and did not develop NC using the t-test and then further examined for independent relationships using multivariate logistic regression. Two regression models were constructed for this purpose. The first examined relations between the development of NC (dependent variable) and urine
sodium (mmol/day), potassium (mmol/day), chloride (mmol/day), calcium to creatinine (mg/mg), and urine volume (ml/day). The second model examined the relationship between NC and plasma sodium (mmol/l), potassium (mmol/l), chloride (mmol/l), ionized calcium, bicarbonate (mg/dl), and whole blood hematocrit (%). These models were repeated for rats grouped based on the presence of severe NC (NC severity category > 3).

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Figure 1.
Von Kossa and Alizarin Red stains of kidney sections from Sprague-Dawley Rats. The panel depicts (a) Von Kossa and (b) Alizarin-Red staining from rats fed a control diet; (c) Von Kossa and (d) Alizarin-Red staining from rats fed a low chloride diet + i.p. furosemide; and (e) Von Kossa and (f) Alizarin-Red staining from rats fed a low sodium & chloride diet. All panels represent 25X magnification.
Figure 2.
24-hour urine calcium excretion categorized by dietary group. Urine calcium excretion expressed as the 24-hour calcium to creatinine ratio (mg/mg). Solid black dots represent a value greater than 1.5 times the interquartile range from the upper quartile value.
Figure 3.
Average daily weight gain by dietary intervention group. Whiskers represent means +/- standard deviation bars. Solid filled black circles with solid line represents rats fed the low chloride diet. Empty triangles with dashed line represents rats fed the low sodium/chloride diet. Open circles with dotted line represents rats fed a control diet and given i.p. furosemide. Solid black diamonds with dashed line represents rats fed a control diet only.
Table 1
Occurrence of nephrocalcinosis by dietary intervention group

|                  | n   | Mild NC (Category 1) | Moderate NC (Category 2–3) | Severe NC (Category 4–5) |
|------------------|-----|----------------------|-----------------------------|--------------------------|
| Control (w/furosemide) | 6   | 0                    | 0                           | 0                        |
| Low Chloride (w/furosemide) | 12  | 1 (8%)               | 3 (25%)                     | 2 (17%)                  |
| Low Sodium/Chloride (w/furosemide) | 18  | 0                    | 3 (17%)                     | 14 (78%)                 |
|                  | 12  | 0                    | 3 (25%)                     | 9 (75%)                  |

NC, nephrocalcinosis.
Table 2

Lab parameters (M (SD)) in Sprague-Dawley rats by dietary intervention group.

|                | Control     | Control + Furosemide | Low Cl− | Low Cl− Furosemide | Low Na+/Cl− | Low Na+/Cl− Furosemide |
|----------------|-------------|----------------------|---------|-------------------|-------------|------------------------|
| n              | 7           | 6                    | 11      | 12                | 18          | 12                     |
| **Plasma**     |             |                      |         |                   |             |                        |
| Na⁺ (mmol/l)   | 137.3 (0.5) | 135.6 (1.1)*,**,†,§  | 118.1 (2.9)*  | 113.9 (4.3)*,** | 116.3 (4.7)*     | 122.2 (4.4)*,**,**†,§  |
| K⁺ (mmol/l)    | 3.7 (0.2)   | 3.1 (0.3)*,**,†,§    | 2.7 (0.5)*      | 2.2 (0.2)*,** | 2.7 (0.4)*,**†     | 2.7 (0.3)*,**†          |
| Cl⁻ (mmol/l)   | 108.7 (1)   | 103.6 (1.7)*,**,**†,§ | 83.3 (3.7)*  | 74.6 (6.3)*,**  | 80.5 (4.4)*,**†   | 86.1 (6.5)*,**†         |
| **24-hr urine**|             |                      |         |                   |             |                        |
| Na⁺ (mmol/day) | 0.43 (0.17) | 0.78 (0.01)*,**,**†,§ | 0.17 (0.11)* | 0.23 (0.11)*      | 0.14 (0.11)*,**† | 0.25 (0.2)              |
| K⁺ (mmol/day)  | 0.48 (0.16) | 0.67 (0.22)*,**,**†,§ | 1.07 (0.51)* | 0.88 (0.34)*      | 0.60 (0.20)*,**† | 0.50 (0.17)*,**†        |
| Cl⁻ (mmol/day) | 0.54 (0.16) | 1.56 (0.19)*,**,**†,§ | 0.31 (0.19)* | 0.39 (0.21)       | 0.19 (0.20)*,**† | 0.29 (0.25)*            |
| Creatinine (mg/dl) | 53.6 (17.9)    | 21.1 (15.6)*,**,**†,§  | 23.5 (13.4)* | 12 (2.4)***      | 15.3 (1.6)*,**,**† | 11.1 (2.9)*,**,**†      |
| Phos:creat (mg/mg) | 2.24 (1.64)    | 0.02 (0.01)*,**,**†,§  | 0.006 (0.004)* | 0.10 (0.14)***    | 4.66 (1.92)*,**,**† | 4.07 (2.99)*,**,**†    |
| Mg:creat (mg/mg) | 0.27 (0.11)    | 0.55 (0.10)*,**,**†,§  | 1.79 (0.56)* | 1.64 (0.64)*      | 0.69 (0.11)***,**† | 0.80 (0.24)*,**,**†    |
| **ABG**        |             |                      |         |                   |             |                        |
| pH             | 7.44 (0.04)  | 7.50 (0.05)*,**,**†,§ | 7.62 (0.13)* | 7.64 (0.03)*      | 7.58 (0.05)*,**,**† | 7.53 (0.14)*†          |
| HCO₃⁻ (mg/dl)  | 30.3 (2.3)   | 32.4 (2.6)*,**,**†,§ | 41.1 (4.9)*    | 51.5 (8.4)*,**  | 45.0 (4.6)*,**,**†   | 38.7 (4.5)*,**,**†      |
| Ionized Ca²⁺   | 1.33 (0.04)  | 1.35 (0.1)*,**,**†,§  | 1.15 (0.03)*  | 1.03 (0.07)***    | 1.09 (0.05)***,**,**† | 1.14 (0.04)*,**,**†     |

* p < 0.05 for comparisons with controls
** p < 0.05 for comparison with Low chloride group
† p < 0.05 for comparison with Low chloride + furosemide group
§ p < 0.05 for comparison with Low sodium/chloride group

(ABG) – Arterial Blood gas, (Na⁺) – sodium, (K⁺) – potassium, (Cl⁻) – chloride, (HCO₃⁻) bicarbonate, (Ionized Ca²⁺) ionized calcium, (Phos:creat) – phosphorus to creatinine ratio, (Mg:creat) – magnesium to creatinine ratio
Table 3
Clinical and laboratory parameters (M (SD)) in Sprague-Dawley rats by dietary intervention group

|                      | Control | Control + Furosemide | Low Cl− | Low Cl− + Furosemide | Low Na+/Cl− | Low Na+/Cl− + Furosemide |
|----------------------|---------|----------------------|---------|----------------------|-------------|--------------------------|
| n                    | 7       | 6                    | 11      | 12                   | 18          | 12                       |
| Weight gain (g/day)  | 6.7 (0.7) | 6 (0.9)**,†,§ | 1.2 (0.4)* | 0.5 (0.3)**,‡ | 2.6 (0.5)**,† | 1.5 (0.4)**,†,§ |
| Hematocrit           | 42 (1)  | 37 (1)**,†,§         | 51 (2)* | 55 (6)**             | 53 (3)*     | 54 (4)**                 |
| Blood Urea Nitrogen (BUN - mg/dl) | 14 (2.2) | 25 (8.2)* | 26.2 (6)* | 33.8 (6.9)** | 30.8 (14.1)* | 47.3 (5.1)**,†,§ |
| Creatinine(mg/dl)    | 0.23 (0.05) | 0.3 (0.1) | 0.36 (0.09)* | 0.45 (0.13)* | 0.38 (0.13)* | 0.35 (0.1)*,† |
| Urine output (ml/day) | 14.4 (10.6) | 18.3 (3.8) | 14 (7.1) | 11.4 (4.6) | 21.7 (7.8)**,† | 14 (4.3)§ |
| Water intake (ml/100g/day) | 12.1 (7.7) | 19.9 (4.1)* | 28.3 (8.3)* | 30.7 (13.1)* | 30.5 (11.5)* | 28.8 (9.3)* |
| Food intake(g/day)   | 15.7 (5.1) | ND                  | ND      | 10.3 (4.2)* | 11.7 (2.3)* | ND                       |

* p < 0.05 for comparisons with controls
** p<0.05 for comparison with Low chloride group
† p < 0.05 for comparison with Low chloride + furosemide group
§ p < 0.05 for comparison with Low sodium/chloride group
(ND) – Not done