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Effect of Dietary Acidification on Kidney Damage Induced in Immature Chickens by Excess Calcium and Infectious Bronchitis Virus[1]

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ABSTRACT Experiments were designed to evaluate the effect of dietary acidification on the development of kidney lesions induced by excess dietary calcium (Ca) and Gray strain infectious bronchitis virus (IBV). Specific pathogen-free (SPF) chicks and SPF chicks inoculated with Gray strain IBV were fed one of three diets: a commercial pullet grower ration (1% Ca); a commercial layer ration (3.25% Ca); or layer ration plus .5% ammonium chloride (acidified layer ration). Gray strain IBV significantly reduced total kidney weights in males, reduced total kidney weight as a percentage of body weight in males, increased the number of gross kidney lesions, and decreased the number of filtering nephrons when compared with uninoculated birds when both groups were fed the grower ration. The layer ration induced a 60% incidence of kidney lesions, caused a significant increase in kidney weight asymmetry ratios, and caused a 25% reduction in the number of filtering nephrons. Acidifying the layer ration significantly reduced the incidence of gross kidney lesions and reduced kidney weight asymmetry ratios, but did not prevent Ca-induced reductions in filtering nephrons.

(*Key words:* calcium, ammonium chloride, glomeruli, kidney damage, infectious bronchitus virus)

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INTRODUCTION

Urolithiasis is a degenerative kidney disease affecting pullets and laying hens. Typical symptoms of urolithiasis include renal atrophy, kidney weight asymmetry, tissue mineralization, fibrosis, significant reductions in the number of filtering nephrons, and the formation of kidney stones in the ureters and collecting ducts (Blaxland *et al.*, 1980; Siller, 1981; Wideman *et al.*, 1983, 1985; Mallinson *et al.*, 1984; Niznik *et al.*, 1985). Field outbreaks of urolithiasis have been associated with excessive dietary calcium (Ca), inadequate dietary available phosphorus (aP), and infectious bronchitis virus (IBV) (Niznik *et al.*, 1985; Wideman *et al.*, 1985).

Recently, we have shown that typical urolithiasis lesions can be induced by feeding 4-week-old pullets a commercial layer ration and exposing them to nephrotropic Gray strain IBV (Niznik *et al.*, 1985). Urolithiasis also can be triggered without known exposure to nephrotropic IBV by feeding young pullets a diet containing excess Ca and low aP (Wideman *et al.*, 1985).

Kidney stones collected from commercial flocks during spontaneous urolithiasis outbreaks, and from an experimental flock fed a high Ca:low aP diet, were shown to be composed of a unique calcium salt of uric acid, Ca urate (Oldroyd and Wideman, 1986). Stones collected from a broiler breeder male had the same composition. Because identical uroliths form in domestic fowl under a variety of conditions, treatments that prevent uroliths from forming when pullets are fed high Ca diets may also be useful for treating most field outbreaks of urolithiasis (Wideman *et al.*, 1985; Oldroyd and Wideman, 1986).

Avian Ca urate uroliths form by gradual accretion of homogeneous mineral (Oldroyd and Wideman, 1986). Pullets fed high Ca:low aP diets excrete urine having an increased pH and high concentrations of Ca (Wideman *et al.*, 1985). Although preformed uroliths composed of sodium urate salts generally are dissolved in human patients by alkalinization of the urine (Sadi *et al.*, 1985), the formation of calcium-based uroliths in humans generally is associated with a defective renal capacity to acidify the urine (Tessitore *et al.*, 1985). Therefore, urinary acidification was recommended as a mechanism to prevent the gradual accretion of Ca urate stones in pullets fed a high Ca diet (N. G. Oldroyd, personal communication).

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The present study was designed to evaluate the effect of dietary acidification on the development of urolithiasis triggered by high dietary Ca and Gray strain IBV. Ammonium chloride was added to the diet to induce metabolic acidosis and increase urinary acidity (Mongin, 1968). Gross kidney lesions were recorded. Microscopic kidney damage was quantified by determining the number of filtering nephrons. Males and females were included in the study to determine if hormonal differences affect the incidence of kidney damage.

MATERIALS AND METHODS

Two hundred male and female specific pathogen-free (SPF) Single Comb White Leghorn chicks were placed in brooders and fed commercial chick starter ration. They were wingbanded and debeaked at 10 days of age. At 30 days of age, the birds were divided randomly into three diet treatment groups. One group was fed a commercial pullet grower ration containing 1% Ca (control ration group). The second group was fed commercial layer ration containing 3.25% Ca and .45% total P (layer ration group). The third group was fed the same commercial layer ration to which .5% NH_4Cl had been added (layer + NH_4Cl group). All birds had *ad libitum* access to food and drinking water throughout the experiment.

At 60 days of age one-third of the birds from each diet treatment group were moved to a separate rearing facility to avoid exposure to Gray strain IBV (SPF groups). Remaining birds were exposed to Gray strain IBV (6th embryo passage; $10^{5.6}$ EID_{50}/ml) by eye drop and nostril inoculation (Gray IBV groups).

The initial experimental design called for birds to remain on their respective diet treatments until they reached 16 weeks of age. However, due to a surge in mortality during the 11th and 12th weeks, all birds were fed commercial pullet grower ration (1% Ca, .4% available P) from the beginning of week 13 to the end of week 16 when the experiment was terminated.

Serum samples were obtained from five birds in each diet treatment group at 56 days of age, prior to exposure to Gray strain IBV. Serum samples also were obtained at 16 weeks of age from five birds in each of the SPF-control diet treatment groups, and from five birds in each of the Gray-IBV diet treatment groups. Virus neutralization tests were performed by a microtest procedure on the serum samples (Wooley *et al.*, 1976).

A glomerular counting technique was used to quantify microscopic kidney damage. Four males were chosen randomly from each of the treatment groups at 16 weeks of age. They were anesthetized, cannulated, volume expanded, and infused with alcian blue as described previously for determining the number of filtering glomeruli (Niznik *et al.*, 1985).

Necropsies were performed on all birds that died during the experiment and on birds that survived until the experiment was terminated. Body weights, gross kidney appearances, and kidney weights were recorded. Kidneys were scored as grossly damaged when one or more kidney divisions where obviously atrophied, when pale calcified areas were found within the kidney tissue, or when kidney stones were present in the ureter.

Student's t test was used to test the significance of intergroup comparisons unless otherwise noted. Values of $P \leq .05$ were considered to be significant.

RESULTS

Feed and water consumption were not directly measured, but equal quantities of layer ration and layer ration plus NH_4Cl feed were bagged at the start of the study, and approximately equal quantities remained when the birds were switched to pullet grower ration at the beginning of Week 13. Layer ration with and without NH_4Cl caused increased water consumption, as was indicated by the frequency with which water containers had to be refilled. Layer ration with and without NH_4Cl also increased the accumulation of highly fluid manure in dropping pans under the cages. After Week 13, when all birds were switched to pullet grower ration, manure fluidity and water consumption for birds in the layer ration and layer ration plus NH_4Cl treatment groups returned to the levels of the control group.

Between the ages of 8 and 16 weeks, 8 birds fed layer plus NH_4Cl and 15 birds fed layer ration died. All but one had gross kidney lesions and evidence of kidney dysfunction (visceral gout). None of the birds fed control ration died during the same period. Ten birds fed layer ration or layer plus NH_4Cl died two to three weeks after they had been inoculated with Gray strain IBV. During the same period, four of the unin-

oculated SPF birds fed layer ration or layer ration plus NH_4Cl died. Mortality ceased immediately after all groups were switched to the commercial pullet grower ration at the beginning of Week 13. Kidney lesion scores, body weights, and kidney weights of birds that died during the study were not included in the first three tables presented here.

Final body weights are shown in Table 1. As expected, males were significantly heavier than females in all diet treatment groups. Gray strain IBV did not significantly alter body weights within treatment groups. However, layer ration and layer ration plus NH_4Cl significantly reduced male and female body weights relative to birds in the control ration group (Table 1).

Gray strain IBV caused a reduction in the kidney weights of males in all diet treatment groups, and this difference was significant for birds fed the control ration and the layer ration plus NH_4Cl (Table 1). Gray strain IBV did not have a significant effect on total kidney weights of females in any of the treatments, suggesting that a hormonal difference exists or that the higher growth rate of males outpaced the compensatory hypertrophic responses of kidneys damaged by IBV. For birds that survived the experiment, the layer ration and layer plus NH_4Cl treatment groups tended to have smaller kidneys than birds in the control ration group, but these differences were not consistently significant (Table 1).

A major effect of Gray strain IBV was to decrease total kidney weight as a percentage of body weight in males (Table 1). For males in the control ration and layer plus NH_4Cl groups, relative total kidney mass was significantly reduced by Gray strain IBV. Diet treatments did not affect relative kidney mass.

Males and females had virtually identical incidences of gross kidney damage within each of the treatment groups. Therefore, in order to focus on the main effects of diet and IBV status, male and female values were pooled in Tables 2 and 3.

The only significant effect of Gray strain IBV on the incidence of gross kidney lesions was seen in the control ration group (Table 2). For the other comparisons, the effects of diet overwhelmed the relatively minor effect of Gray strain IBV. Approximately 60% of the birds in the layer ration group developed gross kidney lesions by 16 weeks of age. These lesions included obvious atrophy, ureteral stone formation, or mineralization of the kidney tissue. The incidence of gross kidney lesions was reduced

TABLE 1. *Body weights and total kidney weights (mean ± SE) and total kidney weight as a percentage of body weight*[1] *for birds that were alive at 16 weeks of age*

Treatments		n	Body weight	Kidney weight	Kidney weight as % of body weight
			(g)		
Control ration					
Male	SPF[2]	15	1,580 ± 53^{a}	12.60 ± .53^{a}	.80 ± .03^{a}
	Gray IBV[3]	19	1,574 ± 27^{a}	9.86 ± .29^{c}	.63 ± .02^{c}
Female	SPF	11	1,165 ± 43^{cd}	9.57 ± .67^{c}	.82 ± .05^{a}
	Gray IBV	14	1,260 ± 34^{c}	9.56 ± .35^{c}	.76 ± .02^{ab}
Layer ration + NH_4Cl					
Male	SPF	13	1,438 ± 29^{b}	11.71 ± .50^{ab}	.81 ± .03^{a}
	Gray IBV	22	1,350 ± 34^{bc}	9.78 ± .33^{c}	.72 ± .01^{b}
Female	SPF	10	1,047 ± 31^{d}	8.60 ± .30^{c}	.82 ± .02^{a}
	Gray IBV	13	1,110 ± 20^{d}	9.50 ± .34^{c}	.85 ± .02^{a}
Layer ration					
Male	SPF	11	1,374 ± 23^{bc}	10.98 ± .36^{b}	.80 ± .03^{a}
	Gray IBV	13	1,325 ± 20^{c}	10.55 ± .47^{bc}	.79 ± .03^{ab}
Female	SPF	8	1,065 ± 41^{d}	9.06 ± .73^{c}	.85 ± .06^{a}
	Gray IBV	13	1,088 ± 41^{d}	9.26 ± .48^{c}	.88 ± .09^{a}

$^{a-d}$Means with different superscripts within each column differ significantly ($P \leq .05$).

[1] Kidney weight/body weight × 100.

[2] SPF = Specific pathogen free; SPF chicks not exposed to challenge.

[3] IBV = Infectious bronchitis virus; SPF chicks inoculated with IBV.

TABLE 2. *Incidence of gross kidney lesions and kidney weight asymmetry ratios for birds that were alive at 16 weeks of age*

Treatments[1]	n	Gross kidney lesions[2]	Heavy/light kidney weight ratio[3]
		(%)	
Control ration			
SPF[4]	26	0^{d}	$1.07 \pm .01^{b}$
Gray IBV[5]	33	6^{c}	$1.08 \pm .02^{b}$
Layer ration + NH_4Cl			
SPF	23	30^{bc}	$1.41 \pm .16^{a}$
Gray IBV	35	31^{b}	$1.22 \pm .07^{ab}$
Layer ration			
SPF	19	68^{a}	$1.74 \pm .25^{a}$
Gray IBV	26	58^{ab}	$1.76 \pm .21^{a}$

$^{a-d}$Means with different superscripts within each column differ significantly ($P<.05$).

[1] Males and females combined.

[2] Number with lesions/total number evaluated (males and females combined). Significant differences were determined with a Z test (Ryan et al., 1985).

[3] Mean (± SE) heavy kidney weight/light kidney weight ratios.

[4] SPF = Specific pathogen free; SPF chicks not exposed to challenge.

[5] IBV = Infectious bronchitis virus; SPF chicks inoculated with IBV.

approximately 50% by the addition of .5% NH_4Cl to the layer ration (Table 2).

Incidence of gross kidney lesions was confirmed quantitatively by calculating kidney weight asymmetry ratios for individual birds (Table 2). For the control group, the heavier kidney of each bird did not exceed the weight of the lighter kidney by more than 10%. For individual birds in the layer ration groups, heavier kidneys exceeded the weight of lighter kidneys by more than 70%. Adding .5% NH_4Cl to the layer ration did not significantly reduce the asymmetry ratio (Table 2).

In spite of relatively consistent overall mean values for total kidney weights relative to body weights (Table 1), the relationship between kidney weight and body weight was poorly predictive for individuals within specific populations (Table 3). For example, only 31% of the variability in kidney weight was associated with

TABLE 3. *Linear regression equations describing the relationships between total kidney weight (Y values) and body weight (X values) for birds that were alive at 16 weeks of age*

Treatments[1]	n	Equation	Relation[2]
SPF Control[3]			
Control ration	26	$Y = .004X + 6.20$	$r = .56, r^2 = .31$
Layer + NH_4Cl	23	$Y = .008X + .53$	$r = .80, r^2 = .64$
Layer ration	19	$Y = .006X + 2.44$	$r = .61, r^2 = .37$
Gray IBV[4]			
Control diet	33	$Y = .002X + 6.55$	$r = .33, r^2 = .11$
Layer ration + NH_4Cl	35	$Y = .007X + 1.07$	$r = .57, r^2 = .32$
Layer ration	26	$Y = .002X + 6.99$	$r = .22, r^2 = .05$

[1] Male and female values combined.

[2] r^2 = Coefficient of determination.

[3] SPF = Specific pathogen free; SPF chicks not exposed to challenge.

[4] IBV = Infectious bronchitis virus; SPF chicks inoculated with IBV.

TABLE 4. *Virus neutralization titers for serum samples collected from uninoculated specific pathogen-free (SPF) birds and birds inoculated with Gray strain infectious bronchitis virus (Gray IBV) for birds that were alive at 16 weeks of age*[1]

Treatments	Bird number				
	1	2	3	4	5
SPF					
Control ration	<8[2]	32	8	<8	24
Layer + NH_4Cl	<8	<8	32	24	24
Layer ration	8	8	<8	<8	96
Gray IBV					
Control ration	768	1024	1024	2048	256
Layer + NH_4Cl	384	768	512	128	256
Layer ration	512	768	192	256	1024

[1] Negative control = 8; positive control = 384. Values >24 were considered to be positive responses.

[2] Reciprocal of neutralizing end point serum dilution.

covariability in body weight for SPF birds in the control ration group (Table 3; r^2 = .31). Coefficients of determination (r^2 values) were higher for SPF diet groups when compared with respective diet groups exposed to Gray strain IBV. Also, the correlation between total kidney weight and body weight was very poor for Gray strain IBV birds raised on layer ration; this treatment was expected to have the most deleterious impact on the kidneys. An unexpected finding was that correlations between kidney weights and body weights were highest when ammonium chloride was added to the diet (Table 3).

Virus neutralization (VN) titers were entirely negative for serum samples obtained at 56 days of age, prior to exposure to Gray strain IBV (data not shown). The VN titers taken at 16 weeks of age are shown in Table 4. For the uninoculated group, some titers suggest possible exposure to IBV. In particular, the 1:96 titer of one bird strongly suggests exposure. Caretakers of these birds were unavoidably in contact with other flocks, but they were not in contact with the Gray strain IBV-inoculated birds during the course of the study. The VN titers of birds inoculated with Gray strain IBV were higher, and are indicative of effective IBV inoculation in this group.

The glomerular counting technique revealed that Gray strain IBV caused a significant reduction in the number of filtering nephrons only in the control ration group (Table 5). Both layer

TABLE 5. *Mean (± SE) glomeruli per kidney and glomeruli per gram kidney weight in surviving 16-week-old Single Comb White Leghorn cockerels, as influenced by diet and inoculation with Gray strain infectious bronchitis virus (IBV)*

Treatments	n[1]	Glomeruli per kidney	Glomeruli per gram kidney weight
Control ration			
SPF[2]	8	452,900 ± 13,700^a	70,100 ± 3,200^a
Gray IBV[3]	8	397,150 ± 22,400^b	78,000 ± 5,700^a
Layer + NH_4Cl			
SPF	4	305,400 ± 26,600^c	58,200 ± 5,100^{ab}
Gray IBV	8	298,000 ± 30,500^c	63,900 ± 5,600^a
Layer ration			
SPF	8	295,000 ± 20,000^c	54,700 ± 4,200^b
Gray IBV	8	321,000 ± 13,600^c	71,500 ± 3,600^a

[a–c] Values with different superscripts within each column differ significantly ($P \leq .05$).

[1] n = Number of kidneys.

[2] SPF = Specific pathogen free; birds not exposed to IBV challenge.

[3] SPF birds inoculated with Gray strain IBV.

ration and layer ration plus NH_4Cl caused a significant reduction in the number of glomeruli per kidney relative to the groups fed the control ration. Apparently, .5% NH_4Cl did not prevent tubular destruction caused by excess calcium in the layer ration (Table 5), but it did decrease the subsequent development of gross kidney lesions and kidney asymmetry (Table 2). Because males were used for glomerular counting, and the kidney weights of males tended to reflect the damage caused by Gray strain IBV and diet treatments (Table 1), the number of glomeruli per gram of kidney weight did not accurately reflect the extent of kidney damage in the present study (Table 5). Kidney weight asymmetry ratios and the total number of glomeruli per kidney continue to be reliable quantitative measures of urolithiasis kidney damage, whereas total kidney weights and kidney weights relative to body weights are less reliable indices of urolithiasis kidney damage.

DISCUSSION

Immature chickens consistently develop typical urolithiasis lesions when they are fed diets containing high Ca:aP ratios as shown by Niznik *et al.* (1985), Wideman *et al.* (1985), and the present study. Chicks raised on high Ca:low aP diets are known to have higher rates of intestinal Ca absorption and higher levels of vitamin D-dependent intestinal Ca-binding protein than do chicks raised on high Ca:normal aP diets (Morrissey and Wasserman, 1971; Norman, 1985). This suggests that high Ca:low aP diets stimulate increased $1,25(OH)_2$ cholecalciferol synthesis, resulting in increased intestinal Ca absorption in spite of elevated dietary Ca intake (Morrissey and Wasserman, 1971). Increased Ca and $1,25(OH)_2$-cholecalciferol act to inhibit the parathyroid glands (Shane *et al.*, 1969; Norman, 1985), resulting in increased urinary Ca excretion (Wideman *et al.*, 1985). In effect, high Ca:low P diets flood the urine with Ca (Wideman, 1986).

Flooding the urine with Ca decreases the number of filtering nephrons per kidney (Table 5), and thus decreases glomerular filtration rates (Niznik *et al.*, 1985; Wideman *et al.*, 1985). Nephron degeneration presumably is caused when Ca urate crystals precipitate and either damage the nephron cells directly or obstruct the nephron lumen. Remaining nephrons then are forced to handle even higher loads of Ca, thereby increasing the opportunity for additional tubular mineralization.

Focal areas of mineralization frequently are seen by gross dissection of kidneys during the development of Ca-induced urolithiasis as shown here and by Wideman *et al.* (1985). Apparently, debris from damaged nephrons accumulates and obstructs the collecting ducts and ureteral branches, causing the upstream nephrons to degenerate, with the necrotic tissue serving as a focal area for additional mineralization. Tissue debris and Ca urates trapped in the ureter solidify into mineral deposits, thereby accounting for the observation that avian ureteral uroliths form by gradual accretion of homogeneous mineral without evidence of an initiating nidus (Oldroyd and Wideman, 1986).

Obstructed collecting ducts, ureteral branches, and ureters cause pressure-induced atrophy of tissue upstream from the blockage. Undamaged kidney tissue undergoes compensatory hypertrophy. Various combinations of localized atrophy and compensatory hypertrophy account for the significant asymmetry of kidney weights in individual affected birds, as shown in Table 2 and by Wideman *et al.* (1983) and Niznik *et al.* (1985).

The cause of the increase in urine pH when pullets are fed a high Ca:low aP ration has not been determined (Wideman *et al.*, 1985). A defect in urinary acidification caused by nephron damage may exist, because inadequate urinary acidification is a common symptom of kidney damage associated with calcium nephrolithiasis (Tessitore *et al.*, 1985). High dietary Ca:P ratios also may alter the electrolyte balance of the diet. Increasing the ratio of positive:negative electrolytes causes metabolic alkalosis, which would increase urinary pH (Mongin, 1968; Cohen and Hurwitz, 1974; Hamilton and Thompson, 1980). Feeding of a semi-synthetic diet which has an imbalance of sodium and potassium levels was reported to induce urolithiasis in broilers and layers (Anonymous, 1983).

Nephrotropic IBV apparently triggers urolithiasis by initially damaging kidney tissue. The virus invades tubule cells, replicates, causes cell death, and provokes interstitial inflammatory responses associated with kidney swelling. Tubular degeneration and necrosis follow. Urate salts precipitate at focal regions of the kidney, either as a consequence of debris plugging the tubules, or because of mineralization of necrotic tubular elements. Mineralized debris then may

accumulate and block the ureter (Heath, 1970; Siller and Cumming, 1974; Purcell *et al.*, 1976; Chong and Apostolov, 1982; Ratanasethakul and Cumming, 1983). The ureter also may be directly damaged, resulting in inflammation, flaccid dilation, and urine stasis if peristalsis is inhibited (Pohl, 1974). Pressure-induced atrophy and postinflammatory atrophy cause reductions in renal mass and scar tissue formation (Chong and Apostolov, 1982). Results of the present study suggest that kidney tissue damaged by IBV mineralizes slowly unless a high calcium diet is fed simultaneously. Mortality ceased when birds inoculated with Gray strain IBV were removed from the layer ration and returned to pullet grower ration.

Laying hens normally have cyclic urinary excretion patterns for Ca and hydrogen ions, depending on the stage of egg formation and on dietary Ca:P ratios (Fussell, 1960; Anderson, 1970; Simkiss, 1970; Wideman, 1986). In the majority of field outbreaks we have investigated, urolithiasis mortality was not noticed until pullets were housed in laying cages and were fed a layer ration. This also was true when pullets were raised on high Ca diets containing elevated quantities of aP (Wideman *et al.*, 1985). Furthermore, it is the cull or nonlaying hens that typically have the greatest incidence of kidney damage during spontaneous outbreaks of urolithiasis, presumably because these hens must excrete large quantities of Ca in their urine. Overconsumption of particulate Ca has been correlated with increased laying hen mortality due to kidney failure (Hamilton *et al.*, 1985). Cumulative evidence therefore suggests that progression from microscopic kidney damage to gross urolithiasis lesions can be accelerated by elevated urinary Ca excretion or cyclic changes in urine pH during the laying cycle.

The results of the present study indicate that the progression from microscopic kidney damage to gross kidney lesions can be partially inhibited by acidifying the urine, to the extent that mortality, gross kidney lesions, and kidney asymmetry are markedly reduced. However, ammonium chloride does not prevent Ca-induced decreases in the number of filtering nephrons (Table 5), indicating that urinary acidification mainly inhibits gross accretions of urolith mineral.

Dietary acidification should not be attempted as treatment for urolithiasis outbreaks in commercial poultry flocks until substantial additional research has been conducted. As shown in the present study, acidified urine may inhibit the initial accretion of Ca urate mineral, but urinary acidification may not dissolve urate stones that already have solidified (Sadi *et al.*, 1985). Hens with solid stones may not benefit from dietary acidification. Other effects of dietary acidification also can have negative consequences that may outweigh benefits for commercial flocks. Ammonium chloride may act as a diuretic (Mueller, 1962), causing water consumption and manure moisture to increase. Birds with damaged kidneys may be unable to withstand the added stress of increased urine flow through their kidneys. Shell quality may decline due to the development of metabolic acidosis (Mongin, 1968). Finally, if urolithiasis causes a reduced renal capacity for hydrogen ion excretion, acidifying the diet would further exaggerate a condition of nephrogenic metabolic acidosis.

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