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ORIGINAL PAPER

Improved methodology to induce hyperoxaluria without treatment using hydroxyproline

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Abstract The use of hydroxyproline (HP) to generate hyperoxaluria in the rat is a problem because it is impossible to separate the effect of oxalate on renal injury from the effects of HP and the large array of metabolic intermediates formed when HP is converted to oxalate. Previously, the Dahl salt-sensitive (SS) and Brown Norway (BN) rat strains were studied to determine genetic control of resistance or susceptibility to HP-induced renal injury and crystal deposition. To develop a better model to induce hyperoxaluria without causing injury from HP metabolites, animals were fed a diet containing various levels of added oxalate (0, 1, 2, 3, or 5%). After 5 weeks rats were killed and the kidneys were removed for microscopic evaluation of tubule changes and crystal deposition. The 3 and 5% oxalate-fed groups had a substantial increase in urine oxalate, about 50 and 140 μmol/g body weight over controls, respectively. Both the SS and BN 3% oxalate-fed animals showed only slightly elevated tubule area and no crystal deposition. However, BN animals fed 5% oxalate had a dramatic increase in their percent tubule areas compared to control BN rats and treated SS rats. Crystal deposition in the kidneys was only observed in the 5% oxalate-fed groups. The BN kidneys demonstrated a threefold higher crystal deposition compared to oxalate-fed SS rats. We conclude that oxalate-supplemented food is a better method of producing hyperoxaluria in the rat than using HP which may introduce metabolic intermediates injurious to the kidney.

Keywords Hyperoxaluria · Kidney injury · Crystal deposition · Urolithiasis

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Introduction

The incidence of urinary tract stone disease continues to increase. The metabolic evaluation of stones in patients has become more sophisticated including more sophisticated algorithms to evaluate the potential of the patient's urine to support crystal formation as a predictive measure of potential stone disease [1]. However, these advances have fallen short in our ability to treat and prevent stone disease, especially calcium oxalate stone disease, the predominant clinical stone presentation. Treatment primarily focuses on changing urine chemistry to increase dilution of the urine or competitive ion chemistry to avoid crystallization.

In addition, calcium oxalate stones can form on calcium phosphate plaques, Randall's plaques, or they may form independent of such nucleating or attachment enhancing structures [2]. Elevated levels of oxalate have been reported to induce kidney tissue damage and we suggest that oxalate-associated injury could be a prominent contributor to the pathologic calcification [3].

We have recently published a study [4] assessing the development of hyperoxaluria, oxalate induced kidney injury, and retention of calcium oxalate crystals in the Dahl salt-sensitive (SS) and Brown Norway (BN) rats as well as a consomic panel of rats in which chromosomes from the

BN rat were transferred onto the SS genetic background [5]. The goal of that study was to identify the chromosomes containing genes that play a role in the physiologic response of the kidney to oxalate. Hyperoxaluria was generated by including trans-4-hydroxy-L-proline (HP) in the rat's drinking water.

The use of HP to generate hyperoxaluria in a genetic study designed to determine which genes are directly involved in susceptibility or resistance to oxalate-related kidney injury and crystal deposition may be inappropriate, because there may be genetic differences in HP metabolism between the SS and the BN rats [6, 7] that could have an unfavorable effect on the physiology of renal cells. Here, we propose that the effect of oxalate on renal injury can and should be separated from the possible effects of HP metabolic intermediates by using oxalate-containing diets to increase urine oxalate levels. This study will demonstrate whether oxalate feeding is suited to maintain specific levels of urine oxalate without the possible complications encountered when using HP.

Materials and methods

Animals

All animal protocols were reviewed and approved by the Clement J. Zablocki, Veterans Administration Animal Care Committee. Experiments were performed on inbred Dahl salt-sensitive (SS/JrHsdMcwi or SS) and Brown Norway (BN/NHsdMcwi or BN) rats. Only male rats were used in this study. Rats were maintained on Teklad 2020 diet modified to contain 0.4% NaCl for control or with 1, 2, 3, or 5% potassium oxalate included in the diet before pelleting (Teklad Custom Diets, Harlan Laboratories, Madison, WI). All diets contained 0.9% calcium.

Experimental protocol

Each group had three rats of each strain on each of the four experimental oxalate diets, plus a no-oxalate control group. The experiment was repeated twice at different times and the results combined for an experimental $n = 6$ for each data point.

Rats were weighed and 24 h urine samples collected once a week for 5 weeks. Urine samples were collected in tubes containing 100 µl 6N HCl as a preservative. Urine was analyzed for oxalate and creatinine levels. Oxalate was measured using an oxalate oxidase assay [8]. Urine oxalate levels are expressed as µmol Oxalate/100 g body weight. Rats were killed by CO_2 asphyxiation and the kidneys were removed and weighed.

Histology

Kidneys were fixed in 10% neutral buffered formalin solution for 24 h, dehydrated, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). Stained sections were examined under incandescent and polarized light for the presence of morphologic changes in renal architecture and for oxalate crystal deposition, respectively. Tubules were evaluated for the presence of necrosis and degree of dilation. Tubular injury and crystal deposition was evaluated quantitatively using computer imaging to provide a measure of injury compared to controls [4].

Quantitative analysis of images

Stained sections, from each rat, were examined under 10× light microscopy and 10 randomly selected areas (from cortex and outer medulla) from each slide (both experimental and control groups) were evaluated for the degree of tubule dilation. In addition, ten randomly selected areas were digitally captured at 10× power using polarized light for the detection of the birefringent calcium oxalate crystal deposits. Images were captured using an Olympus-BX51 microscope with Olympus-DP70 microscope digital camera (Center Valley, PA) and analyzed using Nikon-NIS-Element D image analysis software (Melville, NY). For the calculation of tubular area, the open space within all tubules (per field) was evaluated by applying a threshold to measure the amount of white area versus total image area [4]. Similarly, a threshold was applied to each image to distinguish between the crystals and the background area. Tubule injury or crystal deposition is expressed as the percent of total area of each captured image.

Statistical analysis

Data are expressed as the mean ± SE. Strains were compared using one-way ANOVA followed by post hoc multiple comparisons using Dunnett's.

Results

Figure 1 illustrates the temporal-response of increasing amounts of oxalate in the food on urine oxalate levels in the SS and BN rat strains. No significant difference in urine oxalate levels was observed between the strains at each time point as oxalate concentration in the food increased from 0 to 5%. There was no detectable increase in urine oxalate above controls in the group fed 1% oxalate. The 2% oxalate-fed group showed only a slight

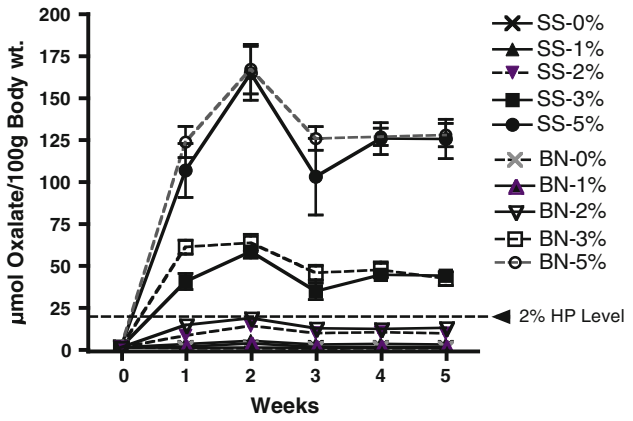

Fig. 1 Weekly urine oxalate levels in SS and BN inbred rat strains in response to varying levels of oxalate in the food for up to 35 days. *Error bars* represent ±SE. ($n = 6$)

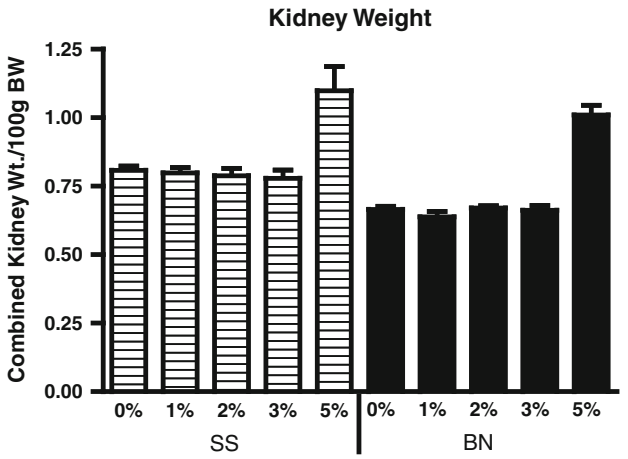

Fig. 2 Kidney wet weight normalized to body weight for SS and BN rats fed various levels of oxalate after 35 days. *Error bars* represent ±SE. ($n = 6$)

elevation in urine oxalate, while the 3 and 5% groups had a substantial increase in urine oxalate, about 50 and 140 µmol/100 g body weight, respectively. The urine oxalate levels were elevated at day 7 (the first time measured) and reached a maximum by day 14. The urine oxalate then decreased slightly and leveled-off for the duration of the study. The horizontal-dashed line in Fig. 1 is the level of urine oxalate achieved in a previous study when rats were given 2% HP in their drinking water [4].

Upon the conclusion of the study, the kidneys were removed for examination. The combined kidney wet weights per 100 g of body weight are shown in Fig. 2. Kidney wet weights were used as a relative measure of kidney hypertrophy. There was no significant increase in the kidney body weight ratio, over controls, except in the 5% oxalate groups. Both the 5% oxalate fed, SS and BN, groups showed a significant increase in kidney weight to body weight ratio over their respective controls.

A quantitative evaluation of tubular injury based on the percent area of tubule dilation between the SS and BN strains is shown in Fig. 3. There was no difference between controls and those animals on the 1 and 2% oxalate diets and are not shown in this graph. Both the SS and BN 3% oxalate-fed animals showed only a slightly elevated tubule area compared to controls. Paralleling their kidney/body weight ratios, both the SS and BN 5% oxalate-fed animals had a dramatic increase in the percent tubule area. The increase in tubule area compared to control for the BN rats fed 5% oxalate was two and one half times as high as the SS rats that were fed 5% oxalate.

Crystal deposition in the kidneys was only observed in the 5% oxalate-fed groups. A quantitative evaluation of crystal deposition (Percentage area occupied by crystals) for both the SS and BN 5% oxalate-fed groups is shown in

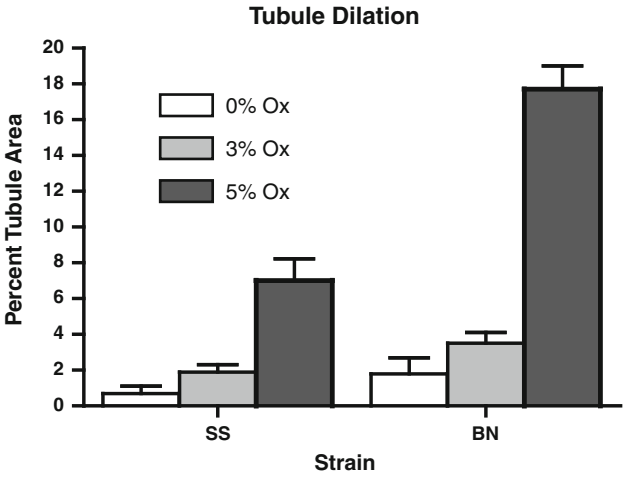

Fig. 3 Quantitative histological evaluation for tubule dilation (indicates injury) of kidney sections of SS and BN strains of rat fed various levels of oxalate. *Error bars* represent ±SE. ($n = 6$)

Fig. 4. BN rats had about three times the level of crystal deposition compared to the SS rats.

Figure 5 shows representative histology images of kidneys from control rats and rats fed 5% oxalate for both the SS and BN strains. Images in Fig. 5a show normal kidneys from the control groups fed base diet with no added oxalate. The images in Fig. 5b show kidneys from the 5% oxalate group and are representative of the images used for the computer analysis of tubule dilation in Fig. 3. The increase in tubule area is apparent in both the SS and BN kidneys compared to controls with the BN tubules having a greater area than the SS kidneys. The images in Fig. 5c are the identical fields of view as in Fig. 5b, except they were taken with polarized light to enhance the visualization of the birefringent crystal deposits. These are representative of the images

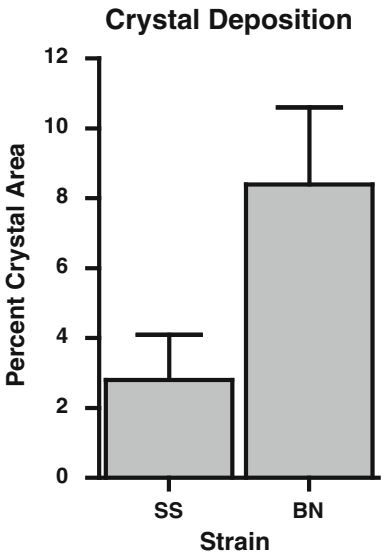

Fig. 4 Quantitative histological evaluation for crystal deposition of kidney sections of SS and BN strains of rat fed 5% oxalate. Crystal deposition was not observed at any other oxalate feeding levels. *Error bars* represent ±SE. ($n = 6$)

used for computer quantification of crystal deposition. The increased crystal deposition in the lumen of the injured tubules can clearly be seen to be greater in the BN kidneys compared to the SS kidneys. Although computer analysis showed a slight increase in tubule dilation in the 3% fed groups, neither tubule dilation nor crystal deposition were visually detectable in any groups other than the 5% oxalate-fed groups and are not shown in Fig. 5.

Discussion

To examine the question of whether oxalate feeding is a suitable method for raising urine oxalate levels in studies of the effect of elevated urine oxalate on kidney urothelial injury, SS and BN rat strains were fed specially prepared diets containing various levels of added oxalate. The SS and BN rat strains were the same as those used in a previous genetic study using HP to induce hyperoxaluria [4].

This study shows that urine oxalate levels can be readily elevated in the rat by the inclusion of supplemental oxalate in the basic rat diet. The urine oxalate levels increased a similar amount in both the SS and the BN strains. No increase in urine oxalate excretion, above control diet groups, was observed until a slight increase was detected in the 2% oxalate supplemented groups. The elevation of urine oxalate levels then continued to increase dramatically as the oxalate supplement increased to 3 and then 5% of the diet.

Supporting the theory that genetics plays a role in the susceptibility to oxalate-induced tubule injury and crystal deposition [4], the BN strain was more susceptible to tubule injury and crystal deposition than the SS strain at the same urine oxalate concentrations. The nature of the tubule injury and crystal deposition, observed microscopically, was the same in this study, using oxalate supplemented food, as was seen in a previous study using HP to increase urine oxalate levels [4]. A most significant finding in this study was that a much higher urine oxalate level was required to induce tubule injury and crystal deposition than was needed in the HP study. This fact accentuates our hypothesis that HP or HP metabolites may enhance or distort the effect of oxalate on kidney injury.

We have used a general measure of kidney injury in this study, quantification of tubule dilation from histology photomicrographs [4], to access the direct comparison of strains under identical dietary conditions. More specific markers of tubule injury and specific parameters of urine composition will be utilized in future studies, examining the mechanism and extent of oxalate injury as these studies progress to more detailed gene mapping. The use of controls at every experimental point using standard histologic techniques have minimized or eliminated any artifacts that could arise.

Despite the observation that the 3% oxalate group was only slightly affected by the increase in urine oxalate compared to the 5% group, the urine oxalate increase was still one-third that achieved by 5% oxalate feeding. It appears that a critical level of urine oxalate needs to be reached before any toxic effects are apparent in the kidney. This raises the question of whether exposure for a longer period of time at sub-toxic urine oxalate levels would result in tubule injury and crystal deposition. Perhaps a more subtle long-term exposure to elevated urine oxalate would provide a more localized limited injury response and crystal deposition more like that observed in human urolithiasis.

We can conclude that oxalate-supplemented food is an excellent method of raising urine oxalate for studies of hyperoxaluria and calcium oxalate stone disease. It eliminates concerns of additional influences on results that may be encountered when using HP or ethylene glycol to increase urine oxalate levels. This is especially of concern in studies determining the genetic basis of susceptibility or resistance to oxalate-induced kidney injury and crystal deposition. Oxalate-supplemented food will be used in all of our future genetic and mechanistic studies on oxalate-induced kidney tubule injury and crystal deposition.

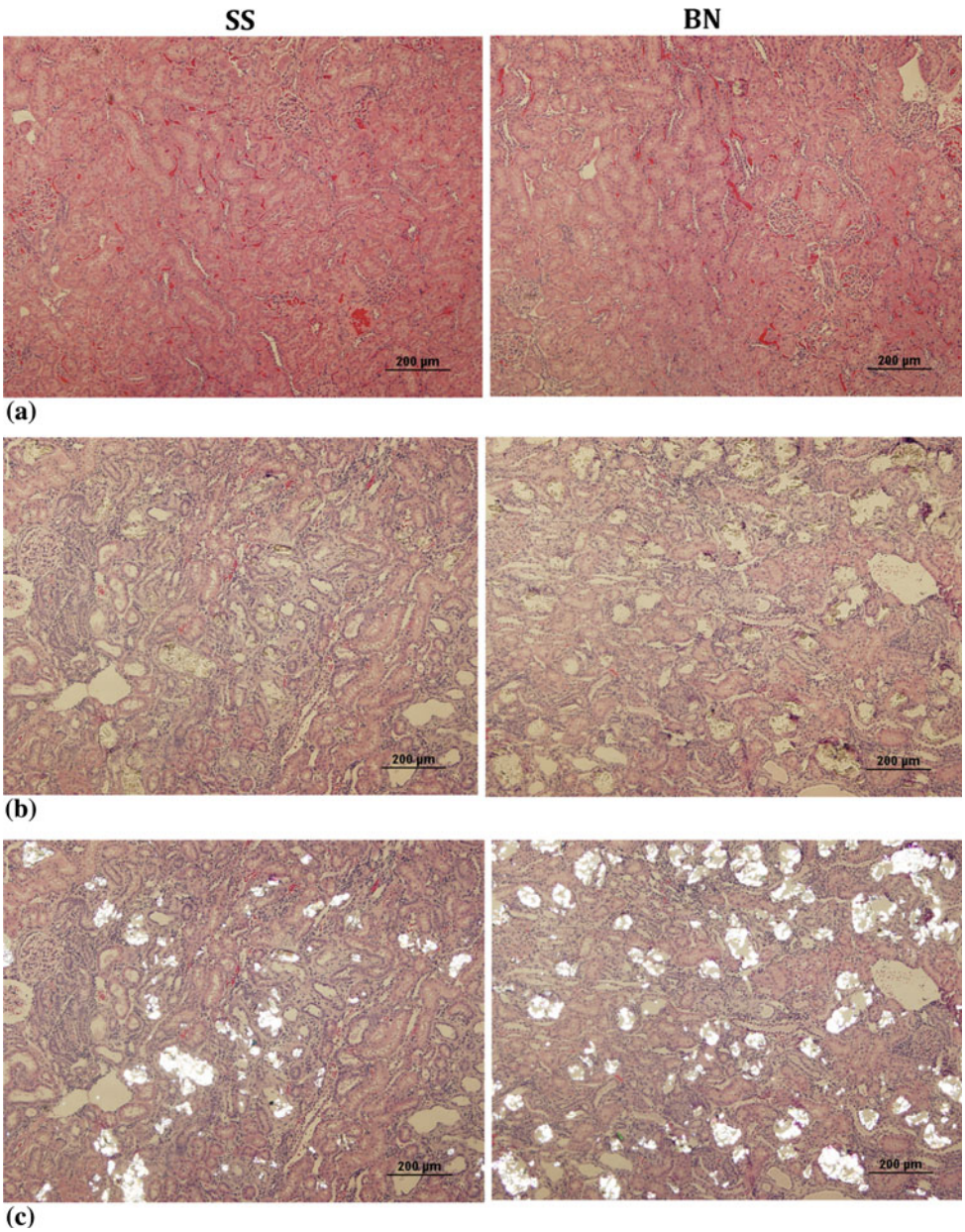

Fig. 5 Representative H&E stained kidney section images from the SS and BN strains at the conclusion of the study. All images were taken with the ×10 objective and the stage calibrated scale bars are presented in μm. **a** Control sections of the SS and BN rats. **b** Kidney sections from 5% oxalate-fed SS and BN rats used to quantify tubule dilation. **c** The identical sections and visual fields as in **b** taken under polarized light to distinguish the birefringent crystal deposits from the background

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