Basic and Translational Science

The Effect of Calcium and Vitamin B6 Supplementation on Oxalate Excretion in a Rodent Gastric Bypass Model of Enteric Hyperoxaluria

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OBJECTIVE	To test the effect of calcium and vitamin B6 therapies on urinary oxalate excretion in a *rodent* model of enteric hyperoxaluria after Roux-en Y gastric bypass (RYGB) surgery.
METHODS	Obese male Sprague-Dawley rats underwent sham ($n = 7$) or RYGB ($n = 10$). Animals were maintained on low oxalate (1.5%) and fat (10%; LOF), normal calcium (0.6 %) diet for 8 weeks and then completed a 2-phase crossover metabolic study. In the first 2-week phase, animals were fed a Low oxalate and fat (LOF), high calcium (2.4%; HC) diet. After a 2-week washout, rats were fed a LOF/normal calcium diet highly enriched with vitamin B6. Urine was collected before and after each intervention. Plasma pyridoxal 5'-phosphate (PLP) and metabolites were measured baseline and 11 weeks after sham or RYGB.
RESULTS	Compared to baseline, sham animals on LOF/HC diet doubled their urinary calcium excretion but not oxalate. RYGB animals on LOF/HC diet decreased urinary oxalate excretion 28% ($P = .001$) without a significant rise in urinary calcium. Vitamin B6 supplementation decreased RYGB urinary oxalate by approximately 15% ($P = .06$), and serum PLP explained 63% of urinary oxalate variability.
CONCLUSION	Based on the findings in this model, calcium supplementation appears to be a reasonable therapy to decrease urinary oxalate in RYGB patients who maintain a low fat and oxalate diet. Serum PLP had a fair correlation to urinary oxalate excretion and may be a useful screening tool in hyperoxaluric RYGB patients. *Further experimental human studies after RYGB are necessary to determine whether these commonly employed supplements truly provide a benefit in enteric hyperoxaluria.* UROLOGY 124: 310.e9−310.e14, 2019. © 2018 Published by Elsevier Inc.

Oxalate is an organic acid found in a variety of plant-based foods (leafy green vegetables) and plant products (chocolate, peanut butter) and is an end-product of liver metabolism. Enteric hyperoxaluria (EH), a pathologic hyperabsorption of dietary oxalate, is a spectrum of gastrointestinal disorders characterized by fat malabsorption (steatorrhea) due to reduced functional small intestinal surface area. As calcium becomes saponified by excessive luminal fat, less calcium is available for oxalate binding, leading to increased intestinal absorption of oxalate via passive or active gut pathways. Roux-en-Y gastric bypass (RYGB), one of the most effective bariatric procedures for sustained weight loss, is the most common cause of EH in the Unites States with RYGB-associated hyperoxaluria as high as 90% in some reports.[1,2]

When a diet low in oxalate and fat is not effective against EH, calcium, and vitamin B6 supplementation have been proposed to reduce kidney stone risk. Increased dietary calcium was tested in the 1970s as a therapy to bind excessive enteric oxalate in EH patients with pancreatitis, ilectomy, or jejunoileal bypass (JIB).[3-6] However, only 1 group has evaluated the effect of calcium supplementation on oxalate excretion in RYGB patients, and this study was limited by short trial duration (13 days) and use of noncommercially available calcium products (effervescent potassium calcium citrate).[7] Vitamin B6, a coenzyme of glyoxalate metabolism, is a well-established therapy to reduce hyperoxaluria in primary hyperoxaluria by increasing liver catalytic activity and oxalate breakdown.[8,9] For stone formers with EH, vitamin B6 is often prescribed at low doses, but this has not been well-studied.[10] Because more data is needed before either of these therapies can be recommended in RYGB patients with EH, we performed a multi-phase crossover

study examining the effect of calcium and vitamin B6 supplementation on urinary stone risk factors and serum metabolites in an established rat model of EH following RYGB surgery.

MATERIALS AND METHODS

Animals and surgical procedures: Animal studies were conducted in accordance with the University of Florida and the NIH Guide for the Care and Use of Laboratory Animals using approved animal protocols. Male Sprague-Dawley rats purchased from Charles River at 3 weeks of age were given free access to water and a high-fat diet (D12492, Research Diets Inc., New Brunswick, NJ) containing 60% fat (lard), 20% protein (casein), and 20% carbohydrate (total 5.2 kcal/gm) for 18 weeks to establish dietary-induced obesity (DIO).
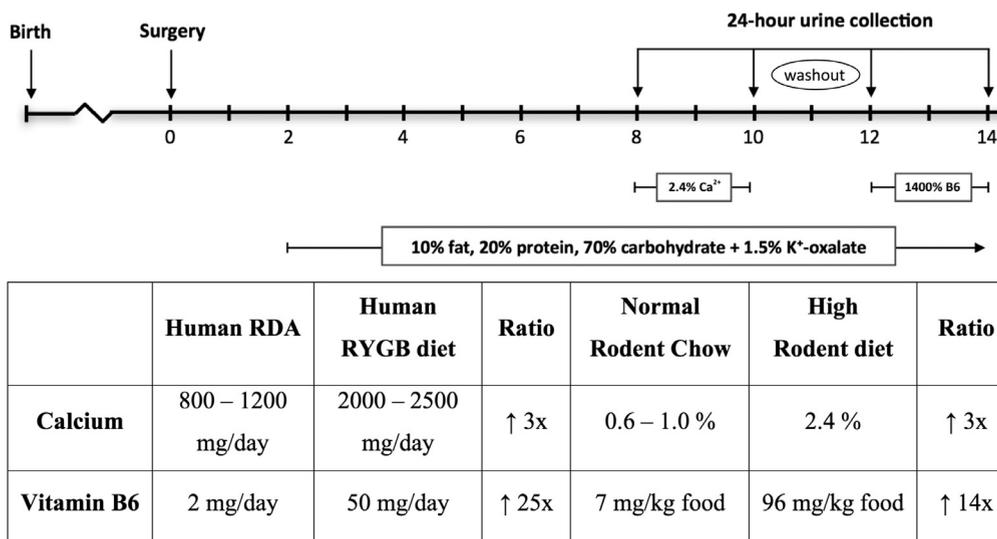
At 20 weeks of age the animals were randomly assigned to either RYGB (*n* = 10) or Sham surgery (*n* = 7). As described previously for RYGB animals,[11] a 4 cm-midline incision was made below the xyphoid process. The terminal ileum was identified at the ileocecal valve and followed orally 35 cm where a 4 mm enterotomy was made to construct the common channel. The jejunum then followed another 10 cm proximally and completely transected to configure the Roux limb. A hand-sewn interrupted end-to-side anastomosis was performed by sewing the proximal portion of biliopancreatic limb (25-35 cm) to the enterotomy using 5-0 Polydioxanone suture (PDS). The vagal nerves of the stomach were identified and mobilized laterally, and the left gastric artery suture ligated. The stomach was then transected 2-3 mm below the level of the gastro-esophageal junction, and an end-to-end hand-sewn gastrojejunostomy was performed using 5-0 PDS, creating a small stomach pouch. The gastrotomy on the defunctionalized stomach was oversewn, fas-

ciae were closed using a running 4-0 Vicryl suture, and the skin reapproximated. All sham animals received a gastric enterotomy, stomach and bowel mobilization, operative time, and closure as RYGB animals.

Dietary regimens: Following their respective procedure, animals were allowed 2 weeks for return of bowel function and were then randomized to Ad Lib 0.6% calcium (normal calcium diet, [NCD]), normal fat (10% fat, 70% carbohydrate, 20% protein; D11032601, Research Diets, New Brunswick, NJ) with 1.5% potassium oxalate supplementation, providing 3.8 kcal/gm (Fig. 1A). Weekly body weights and daily food were recorded for a total of 14 study weeks. Total body weight loss (%) was defined as preoperative weight minus body weight (gm)/preoperative weight (gm).

After 6 weeks on the baseline NCD, animals were placed for 2 weeks on an otherwise identical high-calcium diet (HCD) containing 2.4% calcium. They then underwent a 2-week washout period on the NCD, followed by 2 weeks on the NCD enriched in vitamin B6 (high B6 diet), which contained 1400% (96 mg/kg of food) of the B6 (pyridoxine HCl) contained in the National Research Council's recommended diet for laboratory rats. Rationale for all supplementation amounts is listed in Figure 1B.

Urine collection and B6 serum analysis: Rats were housed individually in metabolic cages and 24-hour urine collections, taken prior to, and at completion of, the 2-week HCD and high B6 diets, were made under 3 mL of hydrated mineral oil placed into vessels containing 20 μL of 2% sodium azide. All animals were pair-fed to minimize food intake effect. Urinary oxalate was determined in acidified (HCl) samples collected from all the animals over a 24-hour period on several occasions using a kit assay (Trinity Biotech #591, St. Louis, MO). Serum levels of pyridoxal phosphate (PLP), the metabolically active form of vitamin B6, were determined by reverse-phase high-performance liquid chromatography with fluorescence

	Human RDA	Human RYGB diet	Ratio	Normal Rodent Chow	High Rodent diet	Ratio
Calcium	800 – 1200 mg/day	2000 – 2500 mg/day	↑ 3x	0.6 – 1.0 %	2.4 %	↑ 3x
Vitamin B6	2 mg/day	50 mg/day	↑ 25x	7 mg/kg food	96 mg/kg food	↑ 14x

Figure 1. **(A)** Animal study workflow from birth to surgery (20 weeks of age) to postoperative week 14. Maintenance on low fat and oxalate diet was maintained postoperative week 2 through week 14 with 2 cross-over phases (high calcium, 2.4%; high B6, 96 mg/kg food). A 2-week washout period occurred between study weeks 10 and 12. **(B)** Human recommended daily allowances (RDA) for calcium and B6 and laboratory rat nutrient requirements in rodent chow. "Supplemented" columns refer to either typical human RYGB supplement doses of calcium and B6 or to supplementation dose amounts used in experimental groups. Ratio column refers to the amount of supplement increase.

Table 1. Roux-en Y gastric bypass 24-hour urine oxalate, calcium and volume for Sham and RYGB animals on baseline and experimental diets. A 2-week washout occurred between HC and baseline B6 diets. Food intake was similar for all animals during the collection periods

	Baseline NC	HC	Baseline B6	High B6
Urine oxalate (umol/d)				
Sham	5.7 ± 2.4	4.8 ± 2.1	5.5 ± 2.0	5.1 ± 1.4
RYGB	16.9 ± 4.9*	**12.2 ± 3.6***	20.0 ± 9.5*	17.1 ± 5.4*
Urine calcium (umol/d)				
Sham	18.1 ± 7.5	33.2 ± 12.0†	16.5 ± 2.9	16.9 ± 1.9
RYGB	24.3 ± 12.7	26.9 ± 15.9	18.6 ± 9.4	18.8 ± 7.6
Urine volume (ml/d)				
Sham	10.6 ± 4.7	21.2 ± 9.8†	15.5 ± 5.1	12.8 ± 3.1
RYGB	30.2 ± 20.4*	**25.2 ± 15.4***	29.8 ± 19.0*	29.4 ± 16.7*

NC, normal calcium; HC, high calcium.
Bold; P <.05 RYGB HC versus RYGB Baseline NC.
All variables shown as mean ± SD.
* P <.001 RYGB versus Sham.
† P <.05 Sham HC versus Sham Baseline NC.

detection before surgical procedure (Fig. 1, time-line point "0") and just before starting high B6 diet (week 11). Aminothiols (homocysteine, cysteine, cysteinylglycine, and glutathione) were quantified as ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate derivatives by reverse-phase high-performance liquid chromatography with fluorescence detection.

Statistical analysis: Mean urine parameters before and after each intervention and between control and RYGB groups were compared using Student's t test for equal variance. P <.05 was accepted as a significant difference.

RESULTS

Body weight: As early as 4 weeks after surgery, total body weight loss (%) in RYGB animals was significantly lower compared to controls (Supplementary Figure 1). By study end, RYGB animals lost approximately 25%-30% of total body weight, paralleling expected human weight loss after RYGB.

Calcium intervention: Table 1 summarizes 24-hour urine oxalate levels before and after HCD interventions for both RYGB and sham rats. Prior to the HCD intervention, after 6 weeks of a baseline NCD diet, the RYGB rat group had mean urine oxalate that was markedly and significantly higher than the control group (16.9 vs 5.7 umol/d; P = .00005) and at all subsequent time-points. There was no significant difference in mean urine calcium excretion (24.3 vs 18.1 umol/d; P = .26715) or calcium-to-creatinine ratios (0.17 vs 0.15; P = .54).

After 2 weeks on the HCD diet, RYGB mean urinary calcium did not change significantly (24.3 vs 26.9 umol/d; P = .20), while the control group's mean urine calcium almost doubled

(18.1-33.2 umol/d; P = .03). RYGB mean urinary oxalate was significantly lower on this diet (16.9-12.2 umol/d; P = .0001) while control group mean urine oxalate did not change significantly (5.7-4.8 umol/d; P = .50).

B6 Intervention: Prior to surgery, mean serum PLP in all rats was 905 ± 86 nmol/L (Table 2). After surgery, mean PLP in RYGB rats decreased to 665 ± 67 nmol/L, a marginally significant difference (P = .06). Serum PLP in sham control rats did not change significantly. Serum homocysteine was significantly higher in postop RYGB rats than baseline or sham controls (P = .001; Table 2) but remained within a normal serum range. Other vitamin B6 metabolites did not show significant changes from pre- to postop.

Table 1 summarizes 24-hour urine oxalate levels before and after high B6 intervention for both RYGB and sham rats. After 2 weeks on a B6-supplemented diet, mean urinary oxalate in RYGB animals declined 15% (20.0-17.1 umol/d; P = .056). No significant change was noted in urinary oxalate for controls (5.5-5.1 umol/d; P = .46). Figure 2 plots serum PLP level (y-axis) from RYGB animals over urinary oxalate (x-axis) excretion prior to B6 supplementation. One RYGB animal was vitamin B6-deficient based on serum PLP cutoff of <350 nmol/L. This animal (Fig. 2, far right/bottom) had the highest urinary oxalate excretion (27.3 umol/d). Based on best-fit line, serum PLP explained 63% of urinary oxalate variation (r^2 = 0.626) at the pre-B6 supplementation time-point.

DISCUSSION

Calcium: We have previously investigated the role of dietary fat, dietary oxalate, and gut colonization with

Table 2. Serum PLP and associated metabolites before and after sham and RYGB procedures

		PLP (nmol/L)	Hcy (umol/L)	Cysteine (umol/L)	Cysteinylglycine (umol/L)	Glutathione (umol/L)
Preop		905 ± 321	3.9 ± 0.8	203 ± 24	1.8 ± 0.4	22.7 ± 4.8
Postop	Sham	732 ± 188	3.7 ± 0.4	189 ± 20	2.4 ± 0.5	26.4 ± 5.2
	RYGB	665 ± 212	5.3 ± 1.0*	189 ± 26	2.1 ± 0.4	24.0 ± 4.6

PLP, pyridoxal phosphate; Hcy, homocysteine.
Variables shown as mean ± SD.
* Significant difference from pre-op value, P <.05.
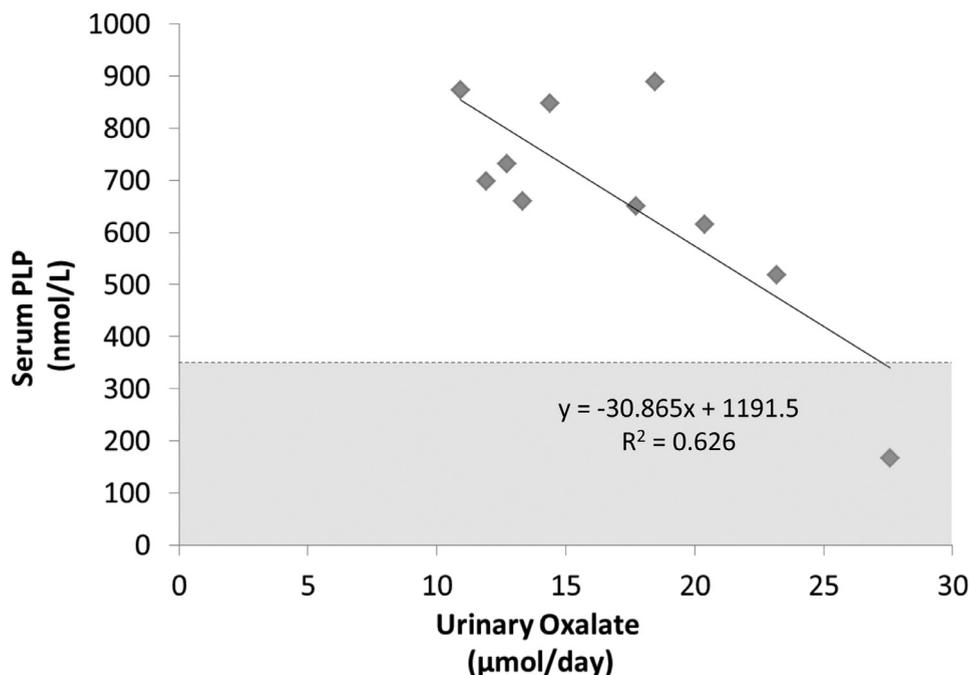
Figure 2. Serum PLP in RYGB animals (week 11) plotted against urinary oxalate prior to supplementation. Values above 350 nmol/L (dotted line, grey area) are considered within normal range.

The chart shows Serum PLP (nmol/L) on the y-axis versus Urinary Oxalate (μmol/day) on the x-axis with the equation $y = -30.865x + 1191.5$ and $R^2 = 0.626$.

Oxalobacter formigenes, an oxalate-metabolizing bacterium, in the DIO RYGB animal model.[11,12] We now demonstrate clinically relevant decreases in urinary oxalate by calcium and B6 supplementation in a model where dietary oxalate is constant and fat is restricted. This is the first and only EH animal model to explore these supplements and directly demonstrate improvement of EH by calcium supplementation. We found that tripling dietary calcium intake in our RYGB animals reduced mean urinary oxalate excretion by 28% ($P = .0001$) without a significant increase in urinary calcium excretion. In matched controls, the higher dietary calcium load was absorbed, filtered, and excreted in the urine as excessive calcium, highlighting the importance of underlying stone pathology when counseling individuals on dietary calcium goals. Total oxalate excretion over time in untreated RYGB animals increased from 16.9 umol/d (week 8)-20 umol/d (week 12, Table 1). This pattern, noted in our previous 2 animal cohorts, seems to peak and find a steady state around week 14—the same week where weight loss nadirs in RYGB (Supplementary Fig. 1).

The 2014 AUA "Guidelines for the Medical Management of Kidney Stones" recommend against routine calcium supplementation in the hyperoxaluric, calcium-stone forming population beyond 1000-1200 mg of dietary calcium/d but acknowledge that higher calcium intake may play a role in patients with EH.[13] These concur with recommendations made by a number of endocrine, obesity, and bariatric surgical societies, endorsing lifelong calcium supplementation in the RYGB population over nutritional concerns.[14] These guidelines dictate daily calcium supplementation ranging from 1200-2000 mg as well

as additional vitamin D supplementation. We modeled our animal calcium dosing (3-fold increase) based on these recommendations and feel that our work lends experimental support to the practice of treating EH patients with calcium supplementation in order to reduce hyperoxaluria and stone risk.

Further clinical evidence of calcium's ability to reduce oxaluria can be seen in patients after JIB, a historical weight loss procedure that was highly malabsorptive. Hylander et al found that a single 2000 mg calcium supplement reduced renal oxalate excretion by 50% (119-60 mg/d) without a significant increase in calcium excretion in 8 JIB patients on a standardized, fixed diet.[5] Another study of 23 JIB patients with steatorrhea reported a significant reduction in oxalate excretion when calcium intake was increased from 250-3000 mg/d.[6] In the only reported study in RYGB patients, Sakhaee et al assessed the urinary response of 24 individuals a mean of 4.7 years after RYGB who ingested an effervescent form of potassium-calcium citrate (40 mEq potassium, 800 mg calcium, 100 mEq citrate/d) for 13 days. This noncommercially available supplement only decreased urinary oxalate levels by 8% but did raise both urine pH and urinary citrate levels to a point where decreases in calcium oxalate agglomeration were noted. The lack of oxalate-binding ability may have been due to lack of standardized participant oxalate intake and inclusion of individuals with normal to moderate range urinary oxalate levels (mean urine oxalate baseline = 42.5 mg/d).

Vitamin B6: B6 (common form: pyridoxine HCl; active derivative: pyridoxal 5-phosphate or PLP) is a versatile, water-soluble vitamin that performs hundreds of

metabolic functions, ranging from production of neurotransmitters to amino acid catabolism.[15] It is also an important cofactor that enhances liver transamination of glyoxylate to glycine. Deficits in this transaminase enzyme cause massive glyoxylate accumulation within the liver, leading to oxalosis and severe hyperoxaluria known as type 1 primary hyperoxaluria (PH1). In addition to hydration and use of crystallization inhibitors, supratherapeutic doses of vitamin B6 have evolved over the last 30 years into the therapeutic standard of care for PH1 individuals until liver transplantation.[16] The overabundance of the PLP cofactor is believed to enhance the activity of the deficient enzyme and shunt the pathway to glycine,[8] a practice that has now been substantiated by a large prospective clinical trial.[9]

As expected for a water soluble vitamin, none of our laboratory animals were B6 deficient prior to their procedure. After RYGB, serum PLP decreased 27% ($P = .06$) while serum homocysteine (an amino acid product of protein metabolism) increased, most likely due to catabolic effects of the procedure and weight loss. One RYGB animal was considered vitamin B6 deficient (1/10 = 10%). This is similar to the 17.6% prevalence of vitamin B6 deficiency reported in a retrospective series of more than 300 individuals 12 months after RYGB.[17] Although this report did not examine other stone forming parameters, it does present a plausible mechanism of hyperoxaluria in this cohort—B6 deficiency after RYGB might slow transamination of glyoxylate to glycine and increases liver oxalogenesis, leading to hyperoxaluria. While RYGB vitamin B6 supplementation had only a marginal effect on urinary oxalate (15% decrease), serum PLP correlated well with urinary oxalate variation ($r^2 = 0.626$) and appeared more reliable in RYGB animals that were hyperoxaluric (Fig. 2). Although these results are intriguing, its effect in our model cannot be definitively established as we were underpowered to detect a difference based on the relatively small magnitude of oxalate change.

Although not as well studied as PH1, vitamin B6 supplementation in individuals with idiopathic or EH has shown clinical promise.[18] In 1988, Nakada et al supplemented 85 idiopathic hypercalciuric stone-formers for 3 months with 60 mg/d of PLP and noted a 24% reduction in urinary oxalate levels—a remarkable finding since most of these individuals did not have high baseline urinary oxalate levels.[19] In a retrospective series of 51 recurrent stone formers with hyperoxaluria (>40 mg/d), Ortiz-Alvarado et al found that dietary advise and pyridoxine (median dose 50 mg/d) reduced mean urinary oxalate by 31% (58-40 mg/d).[20] Notably, the authors excluded 4 patients with EH due to RYGB, Crohn's disease, or small bowel resection. Finally, Mitwalli et al placed 12 recurrent calcium oxalate stone formers with hyperoxaluria (2 with EH) on high dose daily supplementation with 250-500 mg of pyridoxine. After 6 months, mean urine oxalate decreased from 64-42.5 mg/d (38%), a finding that remained statistically significant through study year 1.[21] Although these authors reported no side effects from

such a large dose, neurotoxicity has been reported with rapid dose escalation during supplementation.[22] Because of this, most authors recommend supplementing RYGB patients with resistant hyperoxaluria with 50-100 mg/d (low dose) for 6 months while monitoring for signs of neurotoxicity—then discontinuing the medication entirely.[10] Overall, further studies are necessary to determine if B6 supplementation can create a sustained, meaningful decrease in urinary oxalate and if serum PLP could be a useful nutritional marker for hyperoxaluric RYGB patients.

Our model does have limitations. First, a human would not be able to tolerate an extremely low fat diet such as this (10%) for prolonged periods of time, and higher dietary fat content would be expected to affect calcium and oxalate absorption from the GI tract. Our supplement, calcium carbonate, was mixed into the rodent chow while a RYGB patient would likely take calcium tablets (250 or 500 mg) with small meals. Thus, oxalate absorption could be affected when calcium is delivered as a bolus rather than when mixed with food. Finally, although rat physiology corresponds better to the human condition than mouse, rats have high metabolic rates and may be predisposed to more nutritional abnormalities when using milk-based casein proteins.[23] This could result in oxalate metabolic differences that are not seen in humans. Despite these limitations, we feel our data provide additional validation of the DIO Sprague-Dawley RYGB model as a useful experimental system to investigate EH. Further experimental human studies after RYGB are necessary to determine whether these commonly employed supplements truly provide a benefit in EH.

CONCLUSION

We saw clinically relevant decreases in urinary oxalate by supplementation with calcium in our EH RYGB model where dietary oxalate is constant and fat is restricted. Our results support the guideline recommendations of higher calcium intake for EH patients. Although vitamin B6 did not significantly lower urinary oxalate, serum levels of PLP correlated well with urinary oxalate excretion prior to B6 supplementation and may be a useful screening tool in hyperoxaluric RYGB patients.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.urology.2018.06.061.

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EDITORIAL COMMENT

This manuscript by Canales et al describes the effects of calcium and vitamin B6 supplementation on oxalate excretion in a rat Roux-en-Y gastric bypass model. Medical advances in the treatment of calcium oxalate stones have only made modest gains over the past several decades and the common misconception that excessive calcium in one's diet exacerbates stone formation has been hard to overcome. While this study deals specifically with enteric hyperoxaluria and Roux-en-Y gastric bypass, it does hold similarities between other calcium oxalate stone forming patients. The primary principles proven in this rat model indicate the importance of oral dietary calcium supplementation in patients with excessive oxalate in the gut. In the case of enteric hyperoxaluria, calcium becomes saponified by excessive fat in the intestine and is therefore unavailable to bind to dietary oxalate. In this study, dietary calcium supplementation decreased urinary oxalate excretion by 28% without a rise in urinary calcium. This same principle is often true in many calcium oxalate stone formers. Dietary supplementation with either milk or calcium-fortified orange juice taken with meals is an important treatment to reduce urinary oxalate. Prescribing calcium citrate to your stone forming patient with hyperoxaluria is also beneficial and should be in the armamentarium of all practicing urologists.

Similarly, this study also showed the importance of vitamin B6 (pyridoxine) supplementation, with a 15% reduction in urinary oxalate in these patients. Vitamin B6 is a coenzyme of oxalate metabolism and is an important treatment in primary hyperoxaluria. Clinically, patients with excessive hyperoxaluria can be tested for the disease with genetic testing or a liver biopsy. Practicing urologists should also understand the importance of making this diagnosis in their patients. Oftentimes, oxalate can be lowered by dietary modifications, but in this difficult subset of enteric hyperoxaluria patients, Canales et al show that vitamin B6 supplementation is also effective.

This well-done prospective rat study is important in unraveling the understanding of oxalate metabolism in the interaction between calcium and vitamin B6. It is important that urologists understand that calcium citrate supplementation and vitamin B6 supplementation can and should be utilized in difficult calcium oxalate stone forming patients. The utilization of 24-hour urine collections in patients will help unravel the underlying urinary metabolic problems and confirm the effectiveness of these 2 medications in the patients you treat.

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