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ORIGINAL PAPER

Oxalate synthesis from hydroxypyruvate in vitamin-B6-deficient rats

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Abstract We studied the effects of an intravenous hydroxypyruvate load on endogenous oxalogenesis in rats receiving a standard diet or a vitamin-B6-deficient diet. Twelve male Wistar rats were randomized to two groups and were fed either a standard diet or a vitamin-B6-deficient diet for 3 weeks. Then the animals received an intravenous infusion of 100 mg/ml (960.6 μmol/ml) of hydroxypyruvate slowly over 10 min. Urine samples were collected just before hydroxypyruvate infusion and at hourly intervals until 5 h afterward. Urinary oxalate, glycolate, and citrate levels were measured by capillary electrophoresis. Hourly urinary oxalate excretion peaked within 2 h, while urinary glycolate excretion peaked at 1 h, after the hydroxypyruvate load in both control and vitamin-B6-deficient rats. Both urinary oxalate and glycolate excretion were higher in vitamin-B6-deficient rats than in control rats. Infusion of hydroxypyruvate increased the 5-h urinary oxalate and glycolate excretion to 0.68% (6.56 μmol) and 0.53% (5.10 μmol) of the administered dose (mol/mol), respectively, in the control rats, while oxalate and glycolate excretion, respectively, increased to 2.43% (23.36 μmol) and 0.79% (7.59 μmol) of the dose in the vitamin-B6-deficient rats. Urinary citrate excretion was significantly lower at baseline and all other times in the vitamin-B6-deficient rats than in the control rats. In conclusion, a hydroxypyruvate load increased endogenous oxalate synthesis in control rats, and its synthesis was even greater in vitamin-B6-deficient rats. Vitamin B6 deficiency also resulted in significant hypocitraturia.

Keywords Urinary oxalate · Glycolate · Citrate · AGT · Capillary electrophoresis

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Introduction

Stone formation is a dynamic process that involves crystal formation, aggregation, and growth, and is dependent on the urinary saturation of potential crystal-forming substances [1]. Supersaturation of the urine with calcium oxalate is mainly caused by hypercalciuria and hyperoxaluria [2]. Excessive dietary oxalate intake, increased intestinal absorption of oxalate, and endogenous overproduction of oxalate are considered to be the major causes of hyperoxaluria [3]. Most of the oxalate excreted in the urine is derived from the endogenous metabolism of glyoxylate or ascorbate, while dietary oxalate is partly (0.1–0.2 mmol) absorbed and also makes an important contribution (up to 20% or more of the urinary oxalate excretion) in humans [3, 4]. Almost the entire oxalate load filtered by the kidneys is excreted without significant reabsorption [5]. Oxalate synthesis is mainly dependent on the glyoxylate pathway, and involves enzymatic reactions that convert serine to hydroxypyruvate and other substances [6]. A key step in the glyoxylate pathway is the conversion of glyoxylate to glycine, which is coupled with the conversion of alanine to pyruvate and is catalyzed by a pyridoxal phosphate (vitamin B6)-dependent enzyme, alanine:glyoxylate aminotransferase (AGT) [6–8]. Lack of vitamin B6 causes AGT dysfunction and increases urinary oxalate excretion, leading to hyperoxaluria with hyperglycolic aciduria secondary

to impaired metabolism of the precursors of oxalate [9]. Altered metabolism of several oxalate precursors, including glyoxylate, glycolate, ethylene glycol, and serine, has been reported in vitamin-B6-deficient rats, and deficiency of vitamin B6 augments the conversion of these precursors to oxalate [10]. The sources of glycolate and glyoxylate in stone formers warrant further investigation; so we measured the urinary levels of oxalate, glycolate, and citrate after intravenous administration of hydroxypyruvate to rats with and without vitamin B6 deficiency in the present study.

Materials and methods

Twelve male Wistar rats weighing 180.15 ± 9.70 g (mean ± SD) were acclimatized at the University Animal Center, and were then randomized to two groups of six animals each. One group was fed a standard diet (CE−2; Nihonkurea, Osaka, Japan) and was used as the control group, while the other group was fed a vitamin-B6-deficient diet (Nihonkurea, Osaka, Japan) and was used as the vitamin-B6-deficient group. Both groups received these diets for 3 weeks and both had free access to drinking water during that period. After 3 weeks, the rats were anesthetized with intraperitoneal and subcutaneous urethane (1.2 g/kg body weight), and were hydrated with physiological saline via the femoral vein at a rate of 2.5–3.5 ml/h. Then the animals in both groups were administered 100 mg/ml (960.6 μmol/ml) of hydroxypyruvate as a slow intravenous infusion over 10 min. A total of 1 ml hydroxypyruvate solution was infused to each animal of both groups. Infusion of physiological saline was continued at a rate of 3.5 ml/h throughout the experiment.

The hydroxypyruvate solution for infusion was prepared by dissolving 100 mg (960.6 μmol) of β-hydroxypyruvic acid (molecular weight 104.1; Wako Pure Chemicals, Osaka, Japan) in 1 ml of pure water. The urinary bladder was emptied 1 h before hydroxypyruvate infusion and hourly urine samples were collected by bladder puncture at baseline and every hour until 5 h after infusion. The urinary bladder was completely emptied following each puncture and measurement of the urine volume and pH was done at each time of collection. The samples were immediately stored at −80°C until assay.

Thawed urine samples were filtered through a disposable 0.2 μm filter (Millex-LG syringe-driven unit, Millipore, Bedford, MA, USA), diluted 20- to 40-fold with Milli-Q level pure water obtained using a water purification system (Millipore), and injected into a capillary tube at 50 mbar (5,000 Pa) for 4 s (approximately 20 nl). Then measurement of the urinary glycolate and citrate levels was done by capillary electrophoresis (Agilent CE, Germany) using an organic acid buffer (pH 5.6) for high performance capillary electrophoresis (HPCE) that contained 5 mM 2,6-pyridinedicarboxylic acid with 0.5 mM cetyltrimethylammonium bromide (CTAB) (Agilent Technologies, Germany) [11, 12]. In addition, aliquots of the thawed urine samples were acidified to <pH 2 with 6 N HCl and then were diluted with water in the same fashion to measure the urinary oxalate level by capillary electrophoresis with a pyromellitic acid electrolyte buffer (pH 7.7) for anionic HPCE (Fluka, Switzerland) [11, 12].

The urinary levels of oxalate, glycolate, and citrate measured at 0 h were defined as the baseline values for excretion of each substance. The cumulative increment of urinary oxalate and glycolate excretion above baseline (recovery of the administered dose) after infusion of hydroxypyruvate was calculated for each group by subtracting the baseline oxalate and glycolate values. Hourly urinary excretion of oxalate, glycolate, and citrate was compared with the respective baseline values using Wilcoxon's signed ranks test, while the hourly and total urinary excretion values were compared between groups using the Mann–Whitney test. Data are reported as the mean ± SD and statistical significance was set at $P < 0.05$ for all comparisons.

Results

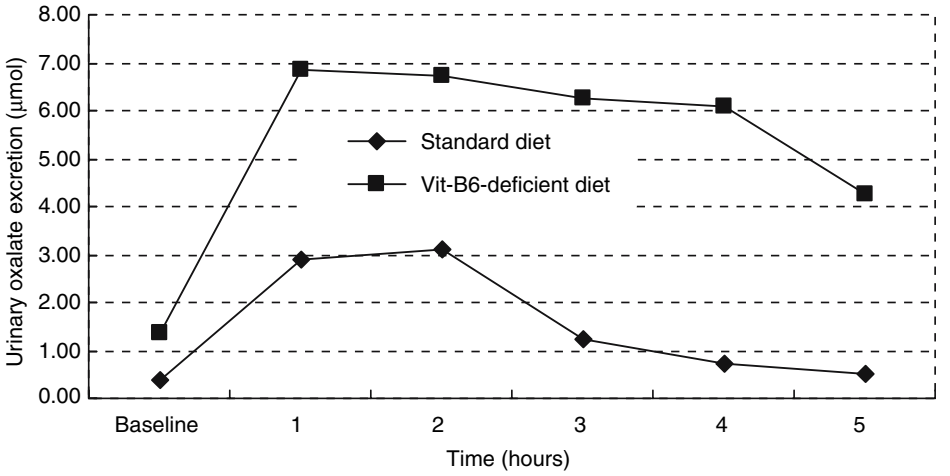
The rats showed significant weight gain during the study from 177.62 ± 6.04 g (mean ± SD) and 182.68 ± 12.45 g at baseline to 403.52 ± 10.13 g and 295.77 ± 26.03 g after 3 weeks in the control and vitamin-B6-deficient groups, respectively ($P < 0.05$ for baseline vs. 3 weeks and for the difference between the two groups at 3 weeks). The vitamin-B6-deficient group showed weight gain of approximately 5.39 g per day, which was significantly lower than the daily increment of approximately 10.76 g in the control group ($P < 0.01$). The weight gain differences between the control and vitamin-B6-deficient diet fed rats in this study were consistent with our previous studies in rats fed with similar diets [9, 13]. However, there were no differences in the behavior of the animals between the two groups. Urinary pH increased significantly with time in control and vitamin-B6-deficient groups but urine volume did not change with time and between groups before and after hydroxypyruvate infusion (Table 1).

Baseline urinary oxalate and glycolate excretion was significantly higher in the vitamin-B6-deficient group (1.37 ± 0.57 and 0.50 ± 0.18 μmol, respectively) than in the control group (0.37 ± 0.10 and 0.23 ± 0.03 μmol, respectively) ($P< 0.01$) (Figs. 1, 2). Hourly urinary oxalate and glycolate excretion peaked within 1–2 h after hydroxypyruvate infusion in both groups (Figs. 1, 2). Hourly

Table 1 Urinary pH and urine volume after administration of β-hydroxypyruvic acid (100 mg) to vitamin-B6-deficient rats and control rats

Groups	Mean pH ± SD					
	Baseline	1 h	2 h	3 h	4 h	5 h
Control ($n = 6$)	5.52 ± 0.08	5.59 ± 0.07[a]	5.63 ± 0.08	5.71 ± 0.03[a]	5.90 ± 0.26[a]	6.13 ± 0.41[a]
Vit-B6-deficient ($n = 6$)	5.46 ± 0.05	5.49 ± 0.11	5.44 ± 0.20	5.52 ± 0.16	5.69 ± 0.23[a]	5.82 ± 0.17[a]
Mean volume ± SD (ml)						
Control ($n = 6$)	0.78 ± 0.36	0.70 ± 0.20	0.57 ± 0.13	0.66 ± 0.31	1.17 ± 0.63	1.32 ± 0.73
Vit-B6-deficient ($n = 6$)	0.55 ± 0.53	0.47 ± 0.21	0.58 ± 0.30	0.81 ± 0.53	1.01 ± 0.41	0.86 ± 0.34

[a] $P < 0.05$ versus baseline



Hourly oxalate excretion	0	1	2	3	4	5	Total	Increment
Control group (μmol)	0.38±0.10	2.87±0.76	3.11±0.79	1.23±0.35	0.70±0.04	0.52±0.07	8.82	6.56
Comparison vs baseline (p value)		0.028	0.028	0.028	0.028	0.046		
Between groups (p value)	0.004	0.109	0.004	0.004	0.004	0.004	0.002	0.002
Vitamin B6 (-) group (μmol)	1.37±0.57	6.84±5.44	6.74±1.71	6.26±2.03	6.10±0.81	4.28±0.74	31.59	23.36
Comparison vs baseline (p value)		0.028	0.028	0.028	0.028	0.028		

Fig. 1 Urinary oxalate excretion after administration of β-hydroxypyruvic acid (100 mg) to vitamin-B6-deficient rats and control rats (*Total* cumulative urinary oxalate excretion from 0 to 5 h; *Increment* cumulative increment of urinary oxalate excretion above baseline from 1 to 5 h after hydroxypyruvate loading)

urinary oxalate excretion was significantly higher than the baseline excretion from 1 to 5 h after infusion in both groups ($P < 0.05$) (Fig. 1), while hourly urinary glycolate excretion was also significantly higher than baseline from 1 to 5 h in both groups, except at 5 h in the control group ($P < 0.05$) (Fig. 2).

Total urinary oxalate excretion (0–5 h) was significantly higher in the vitamin-B6-deficient group than in the control group (31.59 vs. 8.82 μmol, $P < 0.01$) (Fig. 1). The 5-h cumulative increment of urinary oxalate excretion above baseline (recovery rate) accounted for 0.68% (mol/mol) (6.56 μmol) versus 2.43% (23.36 μmol) of the administered dose of hydroxypyruvate in the control group and the vitamin-B6-deficient group, respectively ($P < 0.01$ between groups) (Fig. 1). In the vitamin-B6-deficient group, total urinary oxalate excretion was increased by 3.58-fold (31.59 vs. 8.82 μmol) and the 5-h increment above baseline was 3.56-fold greater than in the control group (23.36 vs. 6.56 μmol) (Fig. 1).

The 5-h cumulative increment of urinary glycolate excretion above baseline (recovery rate) accounted for 0.53% (mol/mol) (5.10 μmol) versus 0.79% (7.59 μmol) of the administered dose of hydroxypyruvate in the control group and the vitamin-B6-deficient group, respectively (Fig. 2). The increase of urinary glycolate excretion after infusion of hydroxypyruvate was greater in the vitamin-B6-deficient group than in the control group, but the difference was not significant (Fig. 2).

Urinary citrate excretion was significantly lower at baseline, as well as from 1 to 5 h after infusion, in the vitamin-B6-deficient group compared with the control group ($P < 0.01$) (Fig. 3). In the control group, hourly urinary

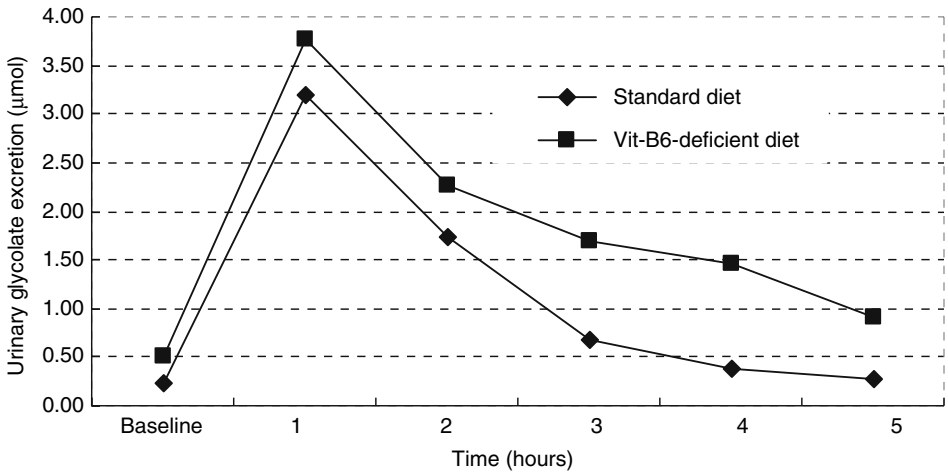


Fig. 2 Urinary glycolate excretion after administration of β-hydroxypyruvic acid (100 mg) to vitamin-B6-deficient rats and control rats (*Total* cumulative urinary glycolate excretion from 0 to 5 h; *Increment* cumulative increment of urinary glycolate excretion above baseline from 1 to 5 h after hydroxypyruvate loading)

Hourly glycolate excretion	0	1	2	3	4	5	Total	Increment
Control group (µmol)	0.23±0.03	3.20±0.41	1.74±0.24	0.68±0.24	0.38±0.11	0.27±0.09	6.51	5.10
Comparison vs baseline (p value)		0.028	0.028	0.028	0.046	0.249		
Between groups (p value)	0.004	0.337	0.337	0.037	0.004	0.004	0.394	0.394
Vitamin B6 (-) group (µmol)	0.50±0.18	3.77±3.19	2.26±0.97	1.69±0.78	1.45±0.48	0.91±0.24	10.57	7.59
Comparison vs baseline (p value)		0.028	0.028	0.028	0.028	0.028		

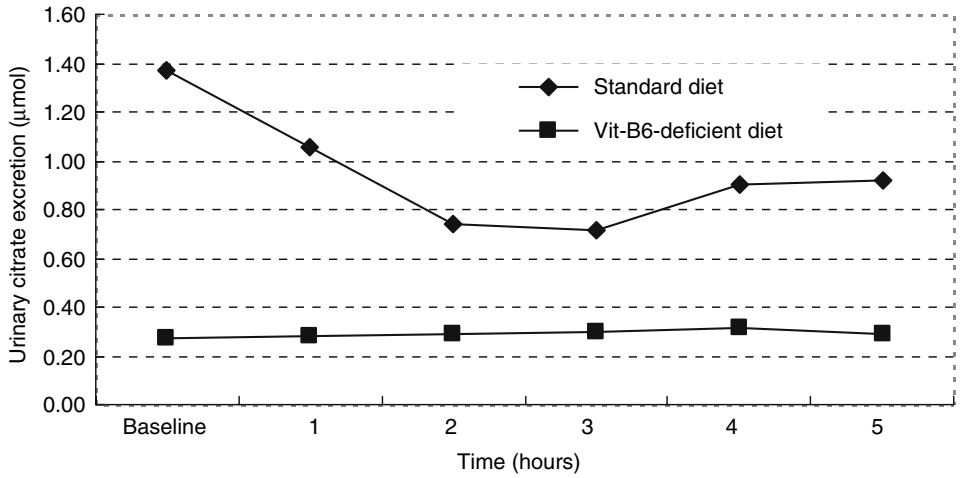


Fig. 3 Urinary citrate excretion after administration of β-hydroxypyruvic acid (100 mg) to vitamin-B6-deficient rats and control rats. (*Total* cumulative urinary citrate excretion from 0 to 5 h)

Hourly citrate excretion	0	1	2	3	4	5	Total
Control group (µmol)	1.37±0.43	1.05±0.12	0.74±0.21	0.71±0.24	0.90±0.44	0.92±0.76	5.70
Comparison vs baseline (p value)		0.116	0.046	0.046	0.173	0.345	
Between groups (p value)	0.004	0.004	0.004	0.01	0.004	0.004	0.001
Vitamin B6 (-) group (µmol)	0.27±0.15	0.28±0.16	0.29±0.10	0.30±0.12	0.32±0.11	0.29±0.08	1.74
Comparison vs baseline (p value)		0.6	0.345	0.345	0.345	0.753	

citrate excretion was significantly lower than the baseline level from 2 to 3 h after hydroxypyruvate infusion ($P < 0.05$) (Fig. 3). In the vitamin-B6-deficient group, however, citrate excretion was low at all times after hydroxypyruvate infusion, but was never significantly different from baseline (Fig. 3).

Discussion

It was previously reported that intravenous infusion of hydroxypyruvate significantly increased hourly urinary oxalate excretion until 3 h after administration in normal rats [14]. Urinary excretion of oxalate, glycolate, and gly-

oxylate showed an increase to 0.4, 0.2, and 0.003% (mol/mol) of the administered dose of hydroxypyruvate, respectively, and the excretion of these three substances peaked at 1 h after administration. In the present study, urinary oxalate and glycolate excretion peaked within 2 h after an intravenous hydroxypyruvate load. In order to assess the stability of hydroxypyruvate we have also measured oxalate level in the hydroxypyruvate solution used in this study, however, the concentration of oxalate in the solution was low, suggesting that the spontaneous generation of oxalate from hydroxypyruvate might be limited. Infusion of 100 mg of hydroxypyruvate caused an approximate 3.5-fold increase of oxalate excretion and an approximate 1.5-fold increase of glycolate excretion in our vitamin-B6-deficient rats compared with the control rats. The hydroxypyruvate load increased urinary excretion of oxalate and glycolate in both the control and vitamin-B6-deficient groups, but its effect was greater in the vitamin-B6-deficient group. In addition, urinary oxalate excretion remained high until 5 h after administration in the vitamin-B6-deficient group, while it returned to baseline within 3 h in the control group. These results suggest that oxalate synthesis was very high in the vitamin-B6-deficient group and that it might take longer (at least, more than 5 h) to excrete all of the oxalate than in the control group. Accordingly, the combination of a hydroxypyruvate load and vitamin B6 deficiency may increase the risk of hyperoxaluria with subsequent calcium oxalate crystal formation, aggregation, and stone growth. On the other hand, baseline urinary citrate excretion was significantly lower in the vitamin-B6-deficient group and it remained low at all times examined. The relatively acidic pH of the urine in the vitamin-B6-deficient group does not seem to be sufficient to explain this hypocitraturia [15]. As Nishijima et al. [9] reported, vitamin B6 acts as a coenzyme for various enzymes of the tricarboxylic acid (TCA) cycle, so citrate metabolism seems to be impaired by vitamin B6 deficiency. Further studies on the activity of various enzymes of citrate metabolism by the effect of vitamin B6 deficiency may provide valuable insight in this regard. Citrate is recognized to be an inhibitor of the nucleation of calcium oxalate and calcium phosphate, as well as inhibiting the processes of crystal formation, aggregation, and stone growth [16–22]. Thus, hypocitraturia associated with vitamin B6 deficiency may be another important risk factor for renal stone formation.

Vitamin B6, a water soluble vitamin, is an essential vitamin for both mental and physiological health in humans. Pyruvate, the anionic form of pyruvic acid, is naturally synthesized in the cells of human body, however, it is also consumed in the diet. The physiological concentration of pyruvate in the blood is 0.6–1 mg/dl (70–114 μmol/l) [23]. Certain foods especially vegetables, fruits (such as red apples), cheese, and dark beer contain high levels of pyruvate. Research has shown that pyruvate administration inhibits the formation of urinary calculi [24, 25]. Various studies have also shown an important role of vitamin B6 in the synthesis of oxalate via the glyoxylate pathway, and it has also been shown that hydroxypyruvate can be converted from serine by the enzyme SPT/AGT [6–8, 26]. Nishijima et al. [9] reported that vitamin B6 deficiency decreases AGT activity and down-regulates AGT gene expression in the liver, resulting in an increased urinary excretion of oxalate and glycolate. A proposed pathway showing the metabolism of oxalate precursors (including hydroxypyruvate) in vitamin B6 deficiency state has also been reported recently [13]. The results of the present study confirm these findings. Dietary sources of serine include meat and dairy products, wheat, peanuts, and soy products [27]. Serine is a nonessential amino acid that is synthesized from other amino acids and metabolites of glucose to participate in protein synthesis, energy production, phospholipid synthesis, and one-carbon unit metabolism (required for RNA and DNA synthesis), and it is known that serine supplies more one-carbon units than any other nutrient. Dietary sources rich in serine include nutritional supplements and all high-protein foods, particularly milk products and meats [27]. Increasing the dietary intake of serine can lead to an increase of hydroxypyruvate in the blood, and may thereby increase urinary oxalate excretion. Further studies of serine loading and measurement of its metabolites might provide more insight into the role of this amino acid. Vitamin B6 deficiency can be associated with many conditions, including malnutrition, bowel disease, and long-term antibiotic therapy [28]. Good dietary sources of pyridoxine include brewer's yeast, eggs, chicken, carrots, fish, liver, kidneys, peas, wheat germ, and walnuts [28].

In conclusion, this study demonstrated that an intravenous hydroxypyruvate load led to a significant increase of urinary oxalate and glycolate excretion in both control and vitamin-B6-deficient rats, but these changes were exaggerated in the vitamin-B6-deficient group. Vitamin B6 deficiency also caused hypocitraturia. Therefore, vitamin B6 deficiency should be included among the risk factors for calcium oxalate stone formation and its supplementation may be recommended in persons with hyperoxaluria and hypocitraturia.

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