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A stable animal model of diet-induced calcium oxalate crystalluria

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Abstract Twenty male Wistar rats, weighing 150 g, were placed in metabolic cages on a 30% sucrose diet for 7 days, before allocation to two groups: a control group ($n = 5$) and a lactose group ($n = 15$). They received respectively a 30% sucrose diet or a 30% lactose diet for 8 weeks, each containing 0.67% calcium and 0.38% phosphorus. After 4 (T1) and 8 (T2) weeks, the serum calcium (Ca) and citrate levels were significantly ($P < 0.01$) higher in rats fed the lactose diet. Serum alkaline phosphatase activity was increased in the lactose group ($P < 0.01$) at T1 and T2. The lactose-rich diet induced an increase in urinary Ca excretion at T1 and T2; citrate excretion was only enhanced at T2 ($P < 0.001$). No difference between the two groups was observed in urinary oxalate (Ox) excretion or creatinine clearance. Crystalluria analysis revealed a marked number (>300/mm^3 at T1 and T2) of calcium oxalate dihydrate crystals (COD) in rats fed the lactose-rich diet, whereas no COD crystals were observed in sucrose-fed control rats at any time point. The formation of COD crystals in lactose-fed rats was related to an increase in calcium oxalate (CaOx) product (pCaOx), which was respectively 12.6 vs 3.9 at T1 and 10.5 vs 1.8 at T2, and an increase in CaOx ratio (Ca/Ox), which was 99.1 vs 7.5 and 67.5 vs 18.5 at T1 and T2, respectively. The high pCaOx and Ca/Ox ratios in the lactose group were due to hypercalciuria, in agreement with the number and the type of crystals. The present experimental model confirms that the ingestion of a 30% lactose diet increases urinary Ca excretion without changing urinary Ox excretion and shows for the first time that it induces a stable and marked crystalluria composed of COD. Such a non-nephrotoxic and stable model is of interest for the study of CaOx crystal formation secondary to hypercalciuria, and thus afterwards eventually for CaOx nephrolithiasis.

Key words Calcium oxalate · Nephrolithiasis · Lactose · Crystalluria

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Introduction

The incidence of renal calcium oxalate (CaOx) calculi is increasing in industrialized countries and crystal formation in urine has been recognized for many centuries as the first step in stone disease. Thus, it appears useful to have a stable experimental model of CaOx crystal formation. A number of experimental models reported to induce CaOx lithogenesis have already been described in the literature, but most of these were associated with nephrotoxicity. For example, a diet containing high concentrations of Ox or Ox precursors such as ethylene glycol induced not only intratubular CaOx crystallization, but also tubular damage and renal failure [3, 8, 18]. On the other hand, it has been demonstrated that the addition to the diet of lactose increased intestinal Ca absorption [30, 32] and urinary Ca excretion in rats compared with sucrose administration [11, 21]. Furthermore, the incorporation of carbohydrates into the diet induced an increased incidence of renal calculi in some studies [4, 27, 31]; however, no such effect was observed by others [28].

In order to obtain a stable non-nephrotoxic model for the study of CaOx crystallization and to investigate the possible effects of excess lactose on the development of uroliths [27], we carried out a study in the rat to determine crystalluria and potential biochemical changes induced by feeding a lactose-rich diet for 2 months.

Materials and methods

Experimental protocol

Twenty male Wistar AF rats, weighing about 150 g, were purchased from Iffa Credo (l'Arbresle, France). During an adaptation period of 7 days, they were fed a diet containing 30% sucrose. They were then randomly allocated into two groups receiving a diet containing either 30% sucrose ($n = 5$; control group) or 30% lactose ($n = 15$) throughout an experimental period of 8 weeks. The compositions of the two diets used are reported in Table 1. The diets contained 0.67% calcium and 0.38% phosphorus (i.e., a calcium/phosphorus ratio of 1.76). Rats were maintained in well-lit rooms with free access to deionized water and food. Animal handling and experimentation conformed to guidelines issued by the European Community as published in the *Journal officiel des communautés européennes* (18 December 1986; L 358). A light/dark cycle provided artificial light from 0700 to 1900 hours. Body weight was recorded weekly.

Each animal was housed in a metabolic cage for 5 days, in order to define baseline state (T0) at the end of the pre-experimental period, and during the fourth (T1) and eighth (T2) weeks of the experimental period. During metabolic study food intake was recorded daily. Urine samples were collected on 1% 2 M HCl over 24 h from day 3 to day 4 of each metabolic time period. Urinary volumes were determined before centrifugation at 3000 *g* for 10 min. Then an appropriate dilution was performed with distilled water in order to allow the measurement of biochemical analytes. In addition, a specific urine collection was made the day after the 24-h collection period, from 1700 to 0900 hours, in order to determine pH and qualitative and quantitative crystalluria. This sample was collected on sodium azide as a preservative and immediately processed for analysis. Blood samples were drawn at the end of the metabolic time period, by subclavian puncture in heparinized vials in rats anesthetized with ethyl-ether for T0 and T1 periods. For the T2 period, blood samples were collected in heparinized vials from the abdominal aorta in rats anesthetized with ethyl-ether; the rats were then killed by exsanguination.

Laboratory determinations

Plasma samples and prediluted 24-h urinary samples were analyzed for Ca (orthocresolphthalein method, Boehringer kit), phosphorus (ammonium molybdate method), urea (enzymatic determination with urease in plasma; Biomérieux kit, orthophthalaldehyde method for urinary samples, Bayer Diagnostic kit) and creatinine (Jaffe method, Boehringer kit) on a multiparameter analyzer Hitachi 717 (Boehringer Mannheim France, Meylan, France). In both samples, citrate concentration was also determined using the citrate lyase enzymatic method (Boehringer kit). Furthermore, total alkaline phosphatase (AP) activity was measured in plasma samples by the kinetic method (Boehringer kit) on a Hitachi 717. Urinary magnesium concentration was determined using the xylidyl method (Boehringer kit) on a Hitachi 717. Finally, urinary Ox concentration was determined by the oxalate oxidase enzymatic method (Biorea kit). Creatinine clearance was evaluated and tubular reabsorption of phosphate (TRP) calculated from the expression:

$$\mathrm{TRP} = 1 - \frac{\text{Urinary phosphate concentration}}{\text{Urinary creatinine concentration}} \times \text{plasma creatinine concentration} \times \text{plasma phosphate concentration}$$

Urinary crystals were analyzed by polarizing microscopy according to the recommendations previously published by Bader et al. [2]. Types of crystals were morphologically identified and numbered in urinary samples specifically collected for such determinations. In these urine specimens, pH was monitored using a pH-meter; Ca and Ox concentrations were determined in order to calculate the CaOx molar product [pCaOx, $(\mathrm{mmol/l})^2$] and the Ca/Ox molar ratio.

Light microscopic examination of rat kidneys

After anesthetizing the animals, kidneys harvested from two rats fed the lactose-rich diet and two controls were analyzed histologically. One fragment of the kidney, frozen in isopentane, was examined by microscopy under polarized light.

Statistical analysis and expression of results

The results have been expressed as means ± SEM throughout. Analysis of variance (ANOVA) was used to test for significant effects of diet.

Table 1 Composition of the two diets

	Sucrose-rich diet (g/100 g)	Lactose-rich diet (g/100 g)
Wheat	53.5	53.5
Wheatgerm	5.0	5.0
Peanut oil	2.5	2.5
$CaCO_3$	1.5	1.5
NaCl	0.5	0.5
Milk casein	7.0	7.0
Sucrose	30	0
Lactose	0	30

Results

Body weight and food intake (Table 2)

Body weight gain was significantly different between rats fed the lactose-rich and the sucrose-rich diet: at the end of the study (T2), the body weight of rats was respectively 439 ± 10.9 g for the control group and 358 ± 10.2 g for lactose group ($P < 0.001$). A significant difference was already observed after 4 weeks of the study. In contrast, daily food intake was similar for the two groups at each time (T1 and T2).

Table 2 Body weight and food intake (mean ± SEM) in rats fed the sucrose-rich versus the lactose-rich diet at the start of the experiment (T0) and after 4 weeks (T1) and 8 weeks (T2)

	T0	T1		T2	
	Sucrose ($n = 20$)	Sucrose ($n = 5$)	Lactose ($n = 15$)	Sucrose ($n = 5$)	Lactose ($n = 15$)
Body weight (g)	149.5 ± 1.6	354.6 ± 6.2	302.1 ± 7.1*	439 ± 10.9	358 ± 10.2*
Food intake (g)	19.5 ± 1.8	22.6 ± 1.2	19.5 ± 1.5	13.1 ± 1.7	13.4 ± 1.2

* $P < 0.001$ between values in rats fed the control sucrose-rich diet and those fed the lactose-rich diet, by analysis of variance

Plasma analytes (Table 3)

The plasma analyte concentrations determined in the two groups of rats at T1 and T2 are shown in Table 3. No significant difference was observed for plasma urea and creatinine concentrations between the two groups after 4 weeks or after 8 weeks of the experiment. Mean plasma Ca concentration was significantly higher ($P < 0.001$) at T1 as well as at T2 in rats fed the lactose-rich diet compared with rats fed the control diet. Mean plasma phosphorus concentration was not significantly different between the two groups throughout the study. A significant effect of the lactose-rich diet on plasma citrate concentration and AP activity was observed. AP activity decreased with age in both groups, but remained significantly higher at T1 and T2 in rats fed lactose than in rats fed the sucrose diet ($P < 0.01$). Plasma citrate concentration was significantly higher in rats fed the lactose-rich diet than in rats fed the sucrose diet at T1 (273 ± 10 μmol/l vs 201 ± 19 μmol/l) as well as at T2 (194 ± 10 μmol/l vs 154 ± 13 μmol/l). However, the percentage differences of Ca and citrate observed between rats fed the lactose-rich diet and rats fed the sucrose diet were similar at T1 (24% for Ca, 36% for citrate) and T2 (18.5% for Ca, 26% for citrate), i.e., a percentage difference of Ca/citrate ratio of 0.67% at T1 and 0.71% at T2.

Urinary analyte excretion (Table 4)

Urinary biochemical analyte outputs, determined at T0, T1 and T2, are presented in Table 4. Urinary Ca excretion after 4 (T1) and 8 weeks (T2) of the experiment was significantly higher (4- to 9-fold) with the lactose-rich diet than with the sucrose-rich diet. Urinary phosphorus excretion was similar in the two groups at T0 and after 4 weeks. After 8 weeks lactose-fed rats excreted lower amounts phosphorus than did rats fed the control diet (273 ± 55 μmol/24 h and 547 ± 25 μmol/24 h respectively; $P < 0.01$). No effect of lactose on Ox excretion was observed. Urinary magnesium excretion was significantly higher in the group fed the lactose-rich diet at T1 ($P < 0.05$) and also at T2 ($P < 0.01$). The urinary excretion of citrate was significantly higher at T2 in the group fed the lactose-rich diet ($P < 0.001$), whereas no significant difference was observed at T1. Urinary creatinine and urea excretion were significantly lower in

Table 3 Plasma analyte concentrations (mean ± SEM) in rats fed the sucrose-rich versus the lactose-rich diet for 4 weeks (T1) and 8 weeks (T2)

	T1		T2	
	Sucrose ($n = 5$)	Lactose ($n = 15$)	Sucrose ($n = 5$)	Lactose ($n = 15$)
Creatinine (μmol/l)	32.4 ± 2.4	36.9 ± 1.4	40.6 ± 3.1	43.1 ± 1.4
Urea (mmol/l)	6.56 ± 0.32	6.29 ± 0.25	5.41 ± 0.21	5.34 ± 0.36
Ca (mmol/l)	2.24 ± 0.08	2.78 ± 0.02***	2.12 ± 0.08	2.51 ± 0.05***
Phosphorus (mmol/l)	2.02 ± 0.22	2.19 ± 0.09	2.07 ± 0.09	2.28 ± 0.05
Citrate (μmol/l)	201 ± 19	273 ± 10**	154 ± 13	194 ± 10*
Total alkaline phosphatase (U/l)	175 ± 5.3	379 ± 38.6**	54.2 ± 5.9	98.5 ± 11.1**

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ between values in rats fed the control sucrose-rich diet and those fed the lactose-rich diet, by analysis of variance

Table 4 Analyte urinary excretion (mean ± SEM) in rats fed the sucrose-rich versus the lactose-rich diet at each collection period (*TRP* tubular reabsorption of phosphate)

	T0		T1		T2	
	Sucrose		Sucrose	Lactose	Sucrose	Lactose
	($n = 5$)	($n = 15$)	($n = 5$)	($n = 15$)	($n = 5$)	($n = 15$)
Excretion						
Calcium (μmol/24 h)	70 ± 24	53 ± 6	69 ± 25	297 ± 54*	32 ± 5.4	287 ± 45**
Phosphorus	927 ± 69	931 ± 31	105 ± 34	111 ± 35	547 ± 25	273 ± 55**
Oxalate	7.75 ± 0.85	9.22 ± 0.44	6.8 ± 0.90	5.4 ± 0.41	4.41 ± 0.47	3.2 ± 0. 33
Magnesium	116 ± 19	117 ± 7	268.5 ± 19.5	390.5 ± 25.8*	185 ± 27.6	264.5 ± 12.8**
Creatinine	31.9 ± 1.1	33.5 ± 0.8	106.8 ± 5.5	90.6 ± 3.4*	129.1 ± 5.7	109.7 ± 4.3*
Urea	6243 ± 338	6198 ± 187	11286 ± 965	8516 ± 271***	9885 ± 349	7594 ± 45**
Citrate	114 ± 27	85 ± 9	163 ± 11	210 ± 30	65 ± 16	222 ± 22***
Creatinine/body weight	0.21 ± 0.01	0.23 ± 0.01	0.30 ± 0.01	0.30 ± 0.01	0.29 ± 0.01	0.31 ± 0.01
Creatinine clearance (μl/s)			36.7 ± 1.3	31.9 ± 1.1	33.7 ± 1.7	31.1 ± 3.5
TRP (%)			98.5 ± 0.44	97.9 ± 0.56	91.5 ± 0.62	95.2 ± 0.98*

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ between values in rats fed the control sucrose-rich diet and those fed the lactose-rich diet, by analysis of variance

Table 5 pH, CaOx molar product (*pCaOx*) and Ca/Ox ratio (mean ± SEM) in rats fed the sucrose-rich versus the lactose-rich diet at each collection period

	T0		T1		T2	
	Sucrose		Sucrose	Lactose	Sucrose	Lactose
	(n = 5)	(n = 15)	(n = 5)	(n = 15)	(n = 5)	(n = 15)
pH	6.54 ± 0.04	6.51 ± 0.03	7.3 ± 0.11	7.09 ± 0.11	7.08 ± 0.06	6.93 ± 0.16
pCaOx	0.72 ± 0.19	0.13 ± 0.09	3.9 ± 1.5	12.6 ± 2.10*	1.8 ± 0.4	10.5 ± 2.2*
Ca/Ox	5.9 ± 1.2	6.9 ± 1.2	7.5 ± 2.4	99.1 ± 17.1**	18.5 ± 5.5	67.5 ± 12.8*

* $P < 0.05$ and ** $P < 0.01$ between values in rats fed the control sucrose-rich diet and those fed the lactose-rich diet, by analysis of variance

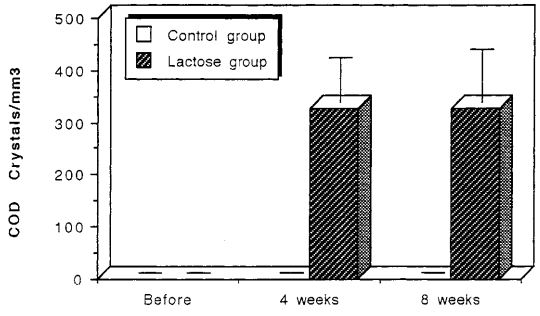

Fig. 1 Mean (+SEM) number of calcium oxalate dihydrate (COD) crystals at T0 and after 4 and 8 weeks in urinary samples of control rats fed the sucrose-rich diet and of experimental rats fed the lactose-rich diet

rats fed the lactose-rich diet at T1 ($P < 0.05$ and $P < 0.001$ respectively) and at T2 ($P < 0.05$ and $P < 0.01$). However, the daily urinary creatinine output per gram body weight and the creatinine clearance were not significantly different between the two groups during the experimental period. Tubular reabsorption of phosphate (TRP) was slightly higher at T2 in rats fed the lactose-rich diet, whereas no difference was observed between the two groups of rats at T1.

Crystalluria

The urinary pH was slightly lower in rats fed the lactose-rich diet than in control rats at each time point, but the difference was not statistically significant (Table 5). Calcium oxalate dihydrate (COD) crystals were absent at the beginning of the study. COD crystals were only observed in rats fed the lactose-rich diet (13 of 15 rats at T1 and 15 of 15 rats at T2), the COD crystal number remaining stable during the experimental period (Figure 1). COD crystals had a bipyramidal shape. Table 5 indicates that the CaOx molar product (pCaOx) was significantly increased after the introduction of the lactose-rich diet, whereas values remained nearly stable in rats fed sucrose during the study. The Ca/Ox molar ratio also increased significantly after the introduction of the lactose-rich diet (99.1 ± 17.1 vs 7.5 ± 2.4 at T1 and 67.5 ± 12.8 vs 18.5 ± 5.5 at T2) .

Calcium citrate crystals were observed at T1 and T2, respectively, in three and two urine specimens from rats fed the lactose-rich diet (different rats at T1 and T2).

As regards other species of crystals observed, amorphous carbonated calcium phosphate (ACCP) granulations were present at T0 in eight samples. In the group fed the lactose-rich diet, eight specimens among 15 had numerous ACCP deposits at T1 and T2, whereas in the control group only a few ACCP crystals were present in five samples. Brushite (calcium hydrogen phosphate) crystals were observed only in rats on the lactose-rich diet: in three rats at T1 (number of crystals: 0.35 ± 0.20/mm^3) and in six rats at T2 (number of crystals: 279.8 ± 248/mm^3).

Histological analysis

On histological analysis the kidneys of controls rats and those fed a lactose-rich diet appeared identical and normal (Fig. 2). Only one calcium deposit was present in one case, but no lesion was found.

Discussion

The present study shows that the ingestion of a 30% lactose diet by the rat induces an early (already after 4 weeks) CaOx crystalluria, in association with a marked increase in urinary Ca excretion. Concomitantly, COD crystals appeared in the urine with a density of 326 ± 86/mm^3 and 328 ± 101/mm^3 after 4 and 8 weeks respectively, i.e., at a level remaining stable throughout the experimental study. In contrast, the ingestion of a 30% sucrose diet (control group) does not induce hypercalciuria or COD crystalluria.

In accord with previous reports of the literature [12, 29], body weight gain was significantly lower in rats fed the lactose-rich diet, although food intake was similar in the two groups. Such an effect has previously been shown with other polyols also, and has been attributed partly to the lipolysis of stored lipids in the polyol-fed rats [13, 14, 23]. The decrease in urinary urea in rats fed

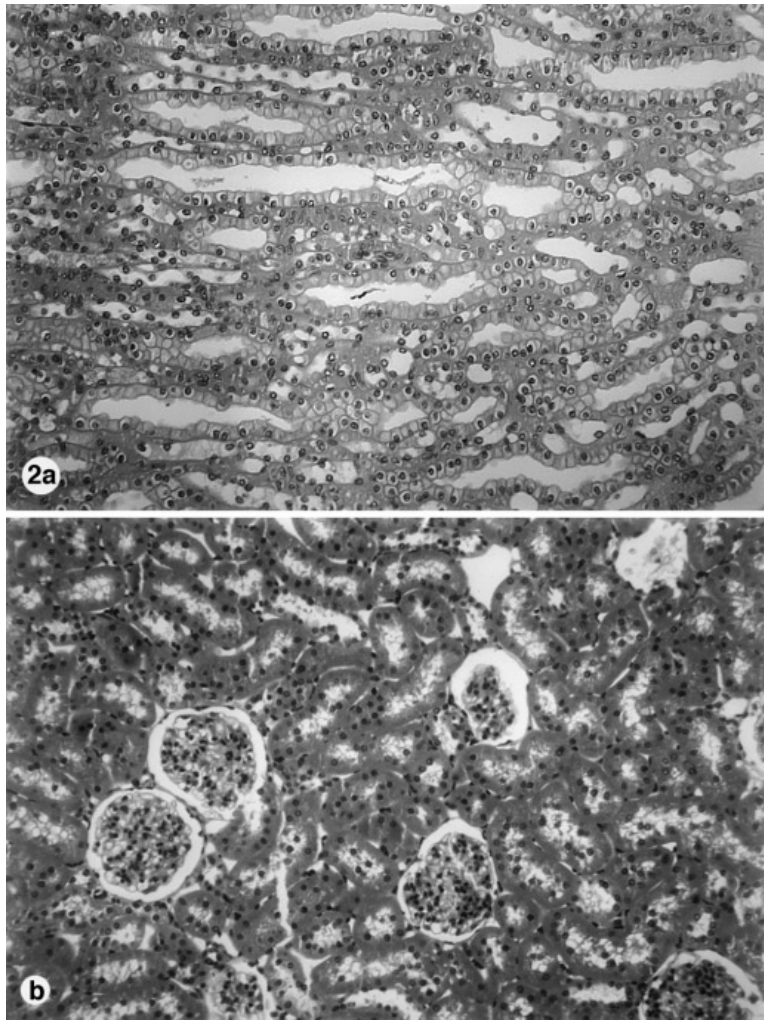

Fig. 2 Light micrographs (×160) of renal medulla (**a**) and renal cortex (**b**) From rats fed the pactose-rich diet.

the lactose-rich diet could also be attributed to the lipolysis induced by this diet. The decrease in creatinine output is related to the decrease in body weight.

Numerous in vivo protocols have been developed in rats to study urinary CaOx formation. Since the majority of urinary calculi contain Ox, it seemed logical to assume that increasing urinary Ox could enhance the risk of stone disease. However, models that used oral administration of a large amount of Ox often led to an alteration in renal function, and in particular tubular damage [3, 8, 17, 18]. In other models, it has been reported that a high animal protein intake increased urinary Ca, Ox and urate excretion, thereby enhancing the risk of stone disease [25].

The originality of the model described in the present study is that the diet containing 30% lactose allows the induction of hypercalciuria in the absence of hyperoxaluria. Many studies have shown that about 50% of CaOx stone-formers exhibit hypercalciuria [6, 24]. Furthermore, the model described here is not nephrotoxic since the creatinine clearance remains unaltered and histological analysis did not reveal any lesions in the kidney.

A significant increase in plasma Ca concentration was observed in rats fed the lactose-rich diet, and consequently urinary Ca excretion was enhanced. This probably reflects a continuous enhancing effect of lactose on intestinal Ca absorption, in agreement with previous studies [21, 29]. The effect of dietary lactose has been attributed to an increased permeability of the intercellular junctions of the intestinal mucosa as a consequence of the increased fluid volume present within the intestinal lumen to maintain isotonicity. Furthermore, using an in vitro model of intestinal transport, such as the Ussing chamber, the absorptive Ca flux has been reported to be increased in the presence of 160 mM lactose via a transcellular pathway, whereas the secretory Ca flux was decreased [10, 20]. A possible role of carbohydrate-induced calciuria in CaOx kidney-stone formation also has been reported in man [22]. After acute oral administration of 100 g glucose or sucrose, the hypercalciuric response of kidney stone-formers and their relatives was greater than that of normal subjects.

An increase in plasma citrate concentration and urinary citrate excretion was observed in our study in rats fed the lactose-rich diet, as reported in previous studies

after carbohydrate or sugar-alcohol administration [14, 15]. These effects have been attributed to an increased generation of intermediates of the citric acid cycle, due to sugar degradation and increased lipolysis. Furthermore, after the peroral administration of a 20% xylitol- or sorbitol-enriched diets to rats, the observed decrease in urinary pH might lead to an increase in the tubular reabsorption of citrate and thereby an increase in plasma citrate level [15]. However, in the present study no variation in urinary pH was observed.

A significant decrease in phosphaturia after 8 weeks associated with a significant increase in tubular phosphate reabsorption was observed in rats fed the lactose-rich diet. The increased plasma Ca concentration would induce a decrease in serum parathyroid hormone (PTH) secretion, which in turn could be responsible for the decrease in phosphaturia. However, the percentage difference in plasma Ca/citrate ratio at 4 and 8 weeks was similar, suggesting that ionized Ca might not have varied. Thus, PTH had been little influenced, if at all. In keeping with this assumption, Kollenkirchen et al. [19] have clearly demonstrated that a 20% lactose-enriched diet, when administered to vitamin-D-deficient rats, was capable of maintaining a normocalcemic state in the absence of a stimulation of PTH secretion.

The optimal length of the study period appeared to be 4 weeks rather than 8 weeks. After 4 weeks there was already marked urinary COD crystal formation in rats fed the lactose-rich diet, with only a slight and non-significant enhancement of citraturia. After 8 weeks, urinary citrate excretion tended to increase significantly, which is less favorable to CaOx crystallization. When increasing the duration of the study further, we have observed that after 4.5 months of the lactose-rich diet, the urinary excretion of citrate continued to increase (up to 7-fold), and thus the COD density returned to a level which was not significantly different from that of the control animals (results not shown).

As has been postulated by several authors, the amount of crystalluria depends on the level of supersaturation above the formation product but also is influenced by the Ca/Ox ratio [5, 26]. In human urine, the Ca/Ox ratio appeared to be the most important factor responsible for the crystalline phases of CaOx observed, and the CaOx product (pCaOx) was the main factor determining the risk of crystallization [9]. The frequency of spontaneous CaOx crystalluria was 5% for pCaOx less than 0.5 $(mmol/l)^2$, and gradually increased up to 100% when pCaOx was more than 3.5 $(mmol/l)^2$. However, in a previous study we concluded that the pCaOx limits that determine CaOx crystallization risk in human urine were not valid for rat urine, because of a higher ionic strength of the latter [7]. Indeed, in the present study, initial pCaOx values were lower than 3 $(mmol/l)^2$ in all animals, and there was no CaOx crystallization. At T1 and T2, pCaOx reached 10 and was stable during the study in rats fed the lactose-rich diet. The pCaOx increase was due to hypercalciuria. Moreover, given that crystal formation depends on the equilibrium between promoting substances and substances protecting against the risk of crystallization, citrate and magnesium excretion did not increase to the same extent as calciuria, according to the number of COD crystals observed. However, in some cases very high urine levels of both citrate and Ca were responsible for Ca citrate crystallization.

Concerning the Ca/Ox ratio, 95.7% of human urine samples contained COM crystals with a Ca/Ox ratio of less than 6. Conversely, with a Ca/Ox ratio higher than 14, nearly 100% of urine samples contained COD crystals [9]. In the present study, we observed a very high Ca/Ox ratio in rats fed the lactose-rich diet, according to the crystal species observed, i.e., COD crystals, which are related to hypercalciuria. Therefore, the present data confirm that COD crystals are favored by a high Ca/Ox ratio and high ionic strength [1, 16].

We conclude that the administration to rats of a diet containing 30% lactose represents a stable model, at least over a time period of 8 weeks, according to present knowledge about metabolic conditions leading to crystalluria and eventually to CaOx stone pathology. This experimental model is devoid of renal toxicity, as demonstrated by a normal creatinine clearance at the end of the experimental period and a normal kidney histological analysis.

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