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Effect of Dietary Supplementation of Vitamin D on Ethylene Glycol-Induced Nephrolithiasis in Rats

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Abstract

Purpose: It is well-recognized that dietary factors may influence in the pathogenesis of urinary lithiasis (UL), the third most prevalent disorder of the urinary tract. Herein we evaluated the effects of the dietary supplementation with vitamin D, which has been recently associated with hypercalcemia, on the occurrence of UL in rats that developed chronic hyperoxaluria after exposition to ethylene glycol, an inducing agent.

Materials and method: Thirty Sprague-Dawley male rats were randomly divided into three groups: Group 1 (control, n=10); Group 2 (0.5% ethylene glycol+vitamin D3, n=10); Group 3 (1.25% ethylene glycol, n=10). Urine samples were collected over a 24 h period at the baseline (day zero) and weekly during four weeks for the dosage of calcium, oxalate, uric acid, citrate and serum creatinine. Animals were euthanized, their right kidneys removed and the corresponding hematoxylin-eosin staining of the histological sections subjected to histological/histomorphometric analyses using the Image J® software. The deposition of calcium salts in the renal parenchyma was quantified by the PIXE technique (Proton Induced X-Ray Emission).

Results: All animals displayed normal levels of serum creatinine (median 0.6 mg/dl) and no statistical difference was found in the daily fluid intake. The volume of the urine samples was significantly higher in animals from G2 when compared to the control animals (10.1 mL versus 4.5 mL, $p<0.05$). Except for hyperoxaluria, which was observed for G3 animals, all the other parameters showed no significant variation after four weeks. In the histomorphometric analysis, nephrocalcinosis was observed for G2 (15 crystals/animal), being the deposition of calcium salts in the renal parenchyma of these animals 100 times higher than the observed for rats from G3 or from the control group.

Conclusion: Although the association of vitamin D3 with ethylene glycol (EG) at 0.5% did not substantially increase the levels of urinary oxalate as observed for EG alone at 1.25%, this association significantly increased the histological damage of the renal parenchyma *via* nephrocalcinosis.

Keywords: Nephrocalcinosis; Lithiasis; Inducing agents; Hyperoxaluria; Vitamin D; Animal model

Introduction

Urinary lithiasis (UL) is the third most prevalent disorder of the urinary tract after urinary infections and prostate disorders [1]. Usually, urinary crystallization occurs due to abnormalities in the composition of the urine, either by an excessive excretion of promoter agents (e.g. calcium, oxalate, uric acid, etc.), by the reduced excretion of inhibitors (such as citrate, magnesium, glycosaminoglycans and Tamm-Horsfall protein), or both [2-4].

Dietary factors are also important for the pathogenesis of urolithiasis since dietary and lifestyle changes may prevent its recurrence [5]. The role of several nutrients as promoters or inhibitors in the formation of urinary stones has been studied, and some nutrients, such as calcium, proteins and sodium, have been extensively characterized as promoters of UL [6]. Recently, abnormalities in the metabolism of vitamin D (or its biologically active form - 1.25-dihydroxyvitamin D) have also been ascribed as important causes of hypercalcemia, which proved to be even more significant than the ingestion of large amounts of calcium [7].

Considering that some of the most common types of kidney stones are composed predominantly of calcium oxalate (CaOx) [8], effective models of chronic hyperoxaluria can be produced in rats through the use of inducing agents, such as ethylene glycol (EG), which promote crystalluria and the consequent deposition of CaOx crystals in the renal parenchyma [9,10]. Additionally, the association between EG and other compounds, such as vitamin D3 (cholecalciferol), can increase the deposition of CaOx in the renal parenchyma and tubules, then resulting in renal lithiasis [11,12]. Herein we evaluated the effect of the

dietary supplementation with vitamin D on the occurrence of UL in rats that developed chronic hyperoxaluria.

Animals and Method

Thirty Sprague-Dawley adult male rats, weighing about 300 g, were randomly distributed into three groups (n=10), and maintained in metabolic cages under controlled lighting and temperature. This study was previously approved by the ethics committee on animal experiments of our institution.

Group 1 (G1) was considered as the "control" and, therefore, no intervention was performed on this group. Animals from group 2 (G2) received ethylene glycol (EG) at 0.5% in their water supply and vitamin D3 (0.5 μM) dissolved in one milliliter of vegetable oil by gavage once daily. Rats from group 3 (G3) received ethylene glycol at 1.25% in their water supply, which was offered "ad libitum". The study was divided into two periods: the initial moment (M0), defined when the supplementation started, and the final moment (M1), when the animals were euthanized, four weeks (28 days) after M0.

During all the experimentation period, urinary volume, water and food intake were measured on daily basis. Urine samples were collected over a 24 h period each week for the dosage of calcium, oxalate, uric acid and citrate. Before freezing, the volume and pH of each urine sample was measured.

Before the euthanasia (M1), when the animals were anesthetized, a sample of urine was collected by bladder puncture for the microbiological analysis, while 3 mL of blood were collected by cardiac puncture for the biochemical dosage of creatinine. Animals were then euthanized with a lethal dose of sodium pentobarbital, and both kidneys were harvested by classical nephrectomy steps via laparotomy. Right kidneys were prepared for the histological analysis and the quantification of nephrocalcinosis. Left kidneys were reserved for the analysis of the deposition of calcium salts in the renal parenchyma.

Histomorphometric and histological quantification of nephrocalcinosis

Right kidneys were fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin (H.E) for histological analysis and histomorphometric quantification of nephrocalcinosis. The paraffin blocks were sectioned to 5 μm thickness, with the sections standardized at the middle aspect of the kidney. For each slide, five random fields were selected and photographed under 40x magnification by a digital camera coupled to a polarized optical microscope. The images were analyzed using a one-hundred-points grid generated by the plug-in of the Image J® program (Figure 1).

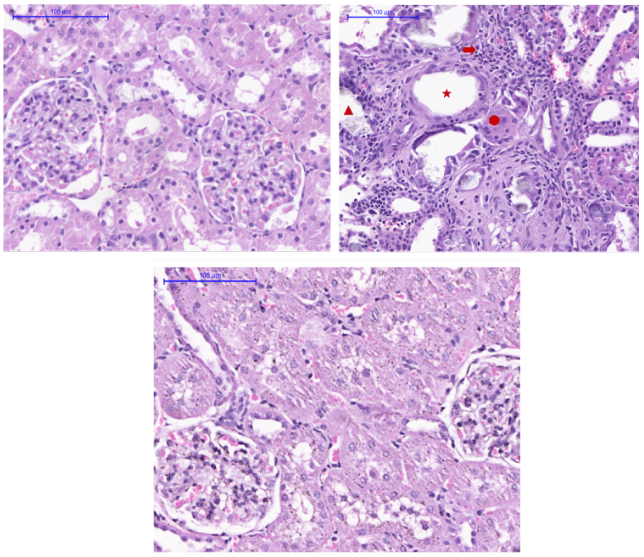

Figure 1: H.E stained section of a control kidney, showing normal organ architecture. H.E stained section of G2 animal: intra-tubular crystals (▲); tubular atrophy (★); acute inflammatory infiltrate (➔) and multinucleated giant cell (●). H.E stained section of a G3 animal: no crystal formation.

Histopathological analysis was performed in a blind analysis by a pathologist, who classified, via optical microscopy, the intensity of the histological parameters into mild, moderate or severe. Parameters under evaluation included tubular atrophy, inflammatory infiltrate intensity and stromal extravasation. The crystals of calcium oxalate (CaOx) in the renal parenchyma were analyzed and counted in five fields for each slide under 40x magnifications, and expressed as the number of renal tubules with crystals.

Calcium quantification in the renal parenchyma

After two days of incubation at 60°C for dehydration, left kidneys were powdered in a crusher at 1070 rpm for five minutes. These lyophilized kidneys were then used for the dosage of calcium (Ca) by the PIXE technique (Proton Induced X-Ray Emission). In this process, the powdered renal tissue was converted into a homogeneous solution using 90 μg of Gallium (Ga) and 1.2 mL of concentrated nitric acid per 0.1 g of renal tissue. Considering the absence of Ga in the original sample, this element was set as the standard for the measurements of Ca. Samples were then placed in the PIXE cam of a Tandem Pelletron 5SDH2 electrostatic accelerator (National Electrostatic Corporation, USA) and irradiated for ten minutes to determine the Ca concentration.

Statistical analysis

The Goodman test was used for contrast among binomial populations [13]. In the study of quantitative variables, analysis of variance in a non-parametric model for two-factor model was used and complemented by the Dunn test [14]. In the tables, lowercase letters were used to indicate statistical significance in the comparisons among groups. Proportions of the same lowercase letter in a referenced

category of response do not differ in the comparison of the groups (p>0.05). All conclusions were made at 5% significance level.

Results

No animals died during the study and all demonstrated a satisfactory weight gain. No statistical difference was found in the daily fluid intake and all microbiological urine cultures were negative. After 28 days (M1), all animals displayed normal levels of serum creatinine (median of 0.6 mg/dl).

Urinary parameters

The volume (UV) of the urine samples collected over a 24 h period was significantly higher in G2 when compared to the control group, and the urinary pH was alkaline, as showed in Table 1. Dosages of urinary citrate, calcium and uric acid did not show significant statistic variation among the different groups at M1. However, G3 animals displayed a persistently higher level of urinary oxalate in comparison to the control group (Table 1).

Urinary Parameter	M1 (28 days)		
	G1	G2	G3
24 h volume (mL)	4.5^{a}	10.1^{b}	4.4^{ab}
pH	9.0^{a}	9.0^{a}	9.0^{a}
Citrate (mg/L)	1.8^{a}	2.2^{a}	3.7^{a}
Uric Acid (mg/dL)	1.3^{a}	1.0^{a}	1.0^{a}
Oxalate (mg/L)	3.6^{a}	5.4^{ab}	11.2^{b}
Calcium (mg/dL)	2.4^{a}	1.2^{a}	1.6^{a}
Serum creatinine (mg/dL)	0.6^{a}	0.6^{a}	0.6^{a}

Table 1: Median of seric and 24 h urinary parameters dosage according to group. *Proportions of the same lowercase letter in a referenced category of response (horizontal lines) do not differ in the comparison of groups (p>0.05). All conclusions were made at 5% significance level. ** mL: milliliters, mg: milligrams; dL: deciliter; L: liter

Histomorphometric evaluation

After 28 days of supplementation with EG at 0.5% and vitamin D3, G2 animals showed a higher prevalence of calcifications, with an average of 15 crystals/animal, as counted by the grid generated using the image J® software. Similarly, histological evaluation by optical microscopy showed 58 intra-tubular crystals/animal for this group. No calcifications were observed for the other groups.

In G2, tubular atrophy was observed for 100% of kidneys at M1 (Figure 1), being not detected for the other groups. Moreover, acute inflammatory infiltrate was classified as moderate in 25% of the G2 animals (Figure 1) but none of the animals from G1 or G3 displayed inflammatory processes in the renal parenchyma.

Calcium dosage in the renal parenchyma

Calcium levels in the renal parenchyma determined by PIXE technique were significantly higher in G2 when compared to the other groups (Table 2).

Group	M1 (28 days)
G1	464 (415/512)a
G2	54935 (48855/61014)b
G3	487 (482/492)a

Table 2: Median and minimum/maximum values of calcium (mg/kg) in the renal parenchyma, according to group, after 28 days. *Proportions of the same lowercase letter in a referenced category of response (columns) do not differ in the comparison of groups (p>0.05). All conclusions were made at 5% significance level.

Discussion

The suggested potential benefits of an adequate ingestion of vitamin D include the control of cellular proliferation and differentiation (impacting in the treatment of cancer), immunomodulation (an important protection factor against infectious and/or autoimmune diseases), and the maintenance of cell membrane fluidity, thus preventing cellular aging [15,16]. However, despite of being essential to our metabolism, excessive ingestion of vitamin D may cause transient negative symptoms or even permanent and irreparable damages [17,18]. Iatrogenic, accidental administration or self-medication is the most common causes of D hypervitaminosis. Conti et al, in a case report concerning vitamin D intoxication, observed severe hypercalcemia and renal failure in a 12 year old boy, admitted for abdominal pain, constipation and vomiting. Plasmatic levels of 25-OH vitamin D were too high and the parathyroid hormone was suppressed [19].

According to some authors, a chronic model of nephrocalcinosis can be obtained in rats through the deposition of CaOx crystals using ethylene glycol (EG) in concentrations above 0.75% [20,21]. Considering these studies, we decided to expose rats to a concentration of EG that is known to induce hyperoxaluria (1.25% EG) and to compare their evolution to animals treated with a lower dose of EG (0.5%). Although theoretically inefficient, this lower concentration of EG would still be able to cause undesirable effects if associated to a potentiating agent [10]. Considering the known interference of vitamin D in the metabolism of calcium, we evaluated the role of this compound as a potentiating agent for EG in this process.

As expected, after 28 days all animals from G3 (1.25% EG) displayed significant levels of hyperoxaluria when compared to the other groups. Nevertheless, CaOx deposition was observed only in G2. We hypothesized that the lower levels of urinary oxalate in G2 could be explained by the higher deposition of urinary crystals in the kidneys of these animals, which correlate with a decreased concentration of these salts in their urine [22].

Urinary volume (UV) is an important factor for the pathogenesis of nephrolithiasis because a low UV may result in an increased concentration of lithogenic solutes and, consequently, in the increased formation of kidney stones [23]. Paradoxically, we observed a higher UV in G2 when compared to the control group, suggesting that this parameter did not influence the CaOx precipitation, differently from previous reports [5,24]. Urinary pH was alkaline (average pH=9.0) and remained stable during the study, suggesting that this parameter did not affected the crystal formation in our model. This observation is in agreement with other studies in which the CaOx solubility was not influenced by the variation of the urinary pH [25,26].

Considering that urinary levels of calcium, citrate and uric acid remained stable in this model, and no statistical difference was observed among the three groups after 28 days of induction, it was not possible to determine the real influence of these parameters on nephrocalcinosis formation.

Some authors have reported an association between the decreased renal function and UL due to urinary obstruction and damage on the renal parenchyma [27]. In this study, no statistical difference in seric creatinine was observed among different groups. Considering that a prolonged exposure to different inducing agents may cause deterioration of the renal function [28], normal levels of creatinine at M1 suggests that it is not a good parameter for kidney function assessment.

According to previous studies, CaOx crystals are formed in the renal tubules and are transported to the interstitial space, causing inflammatory reactions and morphological alterations in the renal architecture [28]. In our study, histopathological analysis showed predominance of acute inflammation, epithelial atrophy and stromal extravasation in G2 animals, which also displayed important nephrocalcinosis. Renal tubular calcifications were significantly higher in G2 when compared to the other groups. Computer analysis performed using the Image J® software demonstrated a clear predominance of CaOx crystals for G2 rats, with a median of 15 crystals per field, while no crystals were observed for animals from the other two groups ($p<0.05$).

Histological findings concerning the calcification process were supported by the calcium dosages in the renal parenchyma via PIXE technique. This method showed a higher concentration of calcium in the renal parenchyma of G2 animals, which was about 100 times higher than the observed for the other groups.

Given these results, in association with the knowledge concerning the physiology of calcium and the influence of cholecalciferol on its metabolism, it can be assumed that the excess of vitamin D can affect the metabolism of calcium, then enhancing its deposition in soft tissues and the subsequent process of calcification. In the kidneys, the severity of the damage depends on the exposure time and the intensity of the process. The final clinical picture may range from the formation of a simple urinary stone to the complete calcification and subsequent destruction of the renal parenchyma, then resulting in the most severe complication of nephrolithiasis: chronic renal failure.

Conclusion

Although the association of vitamin D3 with ethylene glycol (EG) at 0.5% did not substantially increase the levels of urinary oxalate as observed for EG alone at 1.25%, this association significantly increased the histological damage of the renal parenchyma via nephrocalcinosis.

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