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Abstract : Vitamin D prescription in humans should be accompanied by calcium to avoid bone demineralization by Vitamin D receptor signalling. We analyzed whether long-term exposure of rats to vitamin D supplementation, with or without calcium-rich diet, would promote kidney stone formation. Four groups of rats received vitamin D alone (100,000 UI/Kg/3 weeks), a calcium-enriched diet alone, both vitamin D supplementation and calcium rich diet, or a standard diet (controls) during 6 months. Serum and urine parameters and crystalluria have been monitored. Kidney stones have been assessed by 3D-micro-computed tomography, infrared spectroscopy, von Kossa/Yasue staining, and field emission scanning electron microscopy. Although serum calcium levels were similar in the four groups, rats receiving vitamin D had a progressive increase in urine calcium level over the time, especially those receiving both calcium and vitamin D. Calcium alone did not increase urine calcium levels. At 6 months, rats exposed to both calcium and vitamin D, but not rats exposed to calcium or vitamin D alone, developed significant apatite kidney calcifications (mean volume 0.121 mm^3). Overall, co-administration of vitamin D and increased calcium intakes exerted a synergistic role in tubular calcifications or kidney stone formation in this murine model, raising a concern about the cumulative risk of vitamin D supplementation and high calcium intakes in human kidney stone formation.

1. **Introduction**

Vitamin D severe deficiency during infancy leads to rickets and may promote secondary hyperparathyroidism and accelerated bone mass loss in adults (1). Serum 25-hydroxyvitamin D [(25(OH)D] depends on nutritional intakes and absorption of vitamin D2 and vitamin D3 (including supplementation) and solar exposure (2). The synthesis of 1,25-dihydroxyvitamin D [1,25(OH)2D] or calcitriol by the kidney increases calcium absorption and bone remodelling (3). Actually, 1,25(OH)2D binds its receptor (VDR) in enterocytes, increasing the calcium digestive absorption through the transcellular pathway. It has been described for a long time that calcium-dependent kidney stone formers have high 1,25(OH)2D serum levels and/or increased sensitivity to 1,25(OH)2D, possibly due to VDR over-expression or activation (4-6). Bushinsky et al. have extensively studied the main murine model of kidney stone formation, the genetic hypercalciuric stone-forming (GHS) rat, and evidenced an increased expression of VDR in several tissues including gut and bone (6,7).

Vitamin D supplementation, with or without increased calcium intakes, has been proposed for a long time to prevent bone mass loss, especially in post-menopausal women (8). The main goal of vitamin D supplementation is to increase calcium digestive absorption. Since the net bone calcium flux stands near the equilibrium in adults, increased calcium digestive absorption should necessarily result in increased calcium urinary excretion and therefore increase the risk of stone formation. Nevertheless, the role of vitamin D supplementation in kidney stone formation in humans remains controversial. Actually, epidemiological studies did not evidence a link between 25(OH)D serum levels and kidney stone formation or urinary calcium excretion (9-11). One study found no difference in 25(OH)D serum levels between stone formers and control individuals but identified higher 25(OH)D serum levels in hypercalciuric stone formers than in normocalciuric stone formers (12). Another study identified a correlation between 25(OH)D serum levels and urinary calcium excretion in kidney stone formers (13). To date, only one interventional study has been dedicated to the role of vitamin D supplementation on urinary calcium excretion (14). Twenty-nine stone formers received weekly 50,000 IU of ergocalciferol for 2 months. Despite increase of 25(OH)D serum levels, no evidence for a statistical increase in urinary calcium excretion has been observed, although some individuals had increase in urinary calcium excretion (attributed at least for a part to increased protein and salt intakes). The body calcium balance has not been analyzed in this study.

By contrast, the Women Health Initiative (WHI) study is a large double-blind randomized study whose results raise concerns. During an average of 7 years, 36,282 postmenopausal women received 1 gram calcium and 400IU vitamin D daily or a placebo (15). Although no significant reduction in bone fractures has been observed, an increased kidney stone risk has been described in the intervention group, suggesting a cumulative role of calcium and vitamin D supplementation.

We hypothesized that the combination of high calcium intakes and vitamin D supplementation would increase the risk of kidney stone formation and/or kidney tissue calcifications in a murine model. We describe herein the evolution of urine composition and the appearance of kidney stone in rats receiving calcium, vitamin D or both supplementations for 6 months, defining thereby a model of kidney stone disease.

2. Results

2.1 Serum biological parameters

The four groups of rats, "controls", "calcium", "vitamin D" and ""calcium plus vitamin D" had similar baseline serum levels of calcium, magnesium, phosphate, creatinine and urea (Table I). Six months later, no difference has been observed between the four groups with the exception of a small but significant increase of serum creatinine levels in the vitamin D group but not in the calcium plus vitamin D group (Table I). Vitamin D serum levels were significantly higher in the 2 groups of rats receiving vitamin D in comparison to controls (Table I). Rat growth and weight were similar in all groups (not shown).

Table I. Biological parameters, serum

	Controls	Calcium	Vitamin D	Calcium + vitamin D
Baseline				
Calcium, mmol/l	2.51 ± 0.02	2.53 ± 0.03	2.56 ± 0.03	2.45 ± 0.03
Magnesium, mmol/l	0.87 ± 0.02	0.92 ± 0.03	0.83 ± 0.02	0.87 ± 0.03
Phosphate, mmol/l	2.32 ± 0.16	2.64 ± 0.10	2.05 ± 0.18	2.6 ± 0.10
Creatinine, μmol/l	22.5 ± 0.9	19.7 ± 1.0	24.3 ± 1.4	19.4 ± 0.9
Urea, mmol/l	4.97 ± 0.19	5.53 ± 0.51	4.55 ± 0.23	4.8 ± 0.27
At 6 months				
Calcium, mmol/l	2.57 ± 0.04	2.59 ± 0.05	2.67 ± 0.05	2.64 ± 0.03
Magnesium, mmol/l	0.90 ± 0.04	0.94 ± 0.04	1.03 ± 0.09	0.85 ± 0.03
Phosphate, mmol/l	1.92 ± 0.11	1.93 ± 0.09	2.05 ± 0.09	1.94 ± 0.07
Creatinine, μmol/l	27.9 ± 1.3	26.3 ± 1.0	34.2 ± 2.2[a]	28.0 ± 2.1
Urea, mmol/l	4.95 ± 0.29	6.1 ± 0.55	5.07 ± 0.21	5.72 ± 0.34
Vitamin D, ng/ml	55.2 ± 17.9	49.9 ± 1.7	126.2 ± 9.8[a]	110.8 ± 21.7[a]

[a]$P < 0.05$ versus control and calcium groups at 6 months.

2.2 Crystalluria

Crystalluria revealed the frequent presence of calcium oxalate and calcium phosphate crystals in the fresh urine of rats receiving vitamin D and vitamin D plus calcium (Figure 1A-C). Overall, crystals were more frequently present in the urine of rats receiving calcium plus vitamin D than in rats receiving vitamin D only. During the first weeks, crystals were mainly calcium oxalate dihydrate (Figure 1B), whereas calcium phosphate crystals predominated after 2 months (Figure 1C). Urinary pH remained alkaline, around 7, in all groups during the whole protocol (not shown).
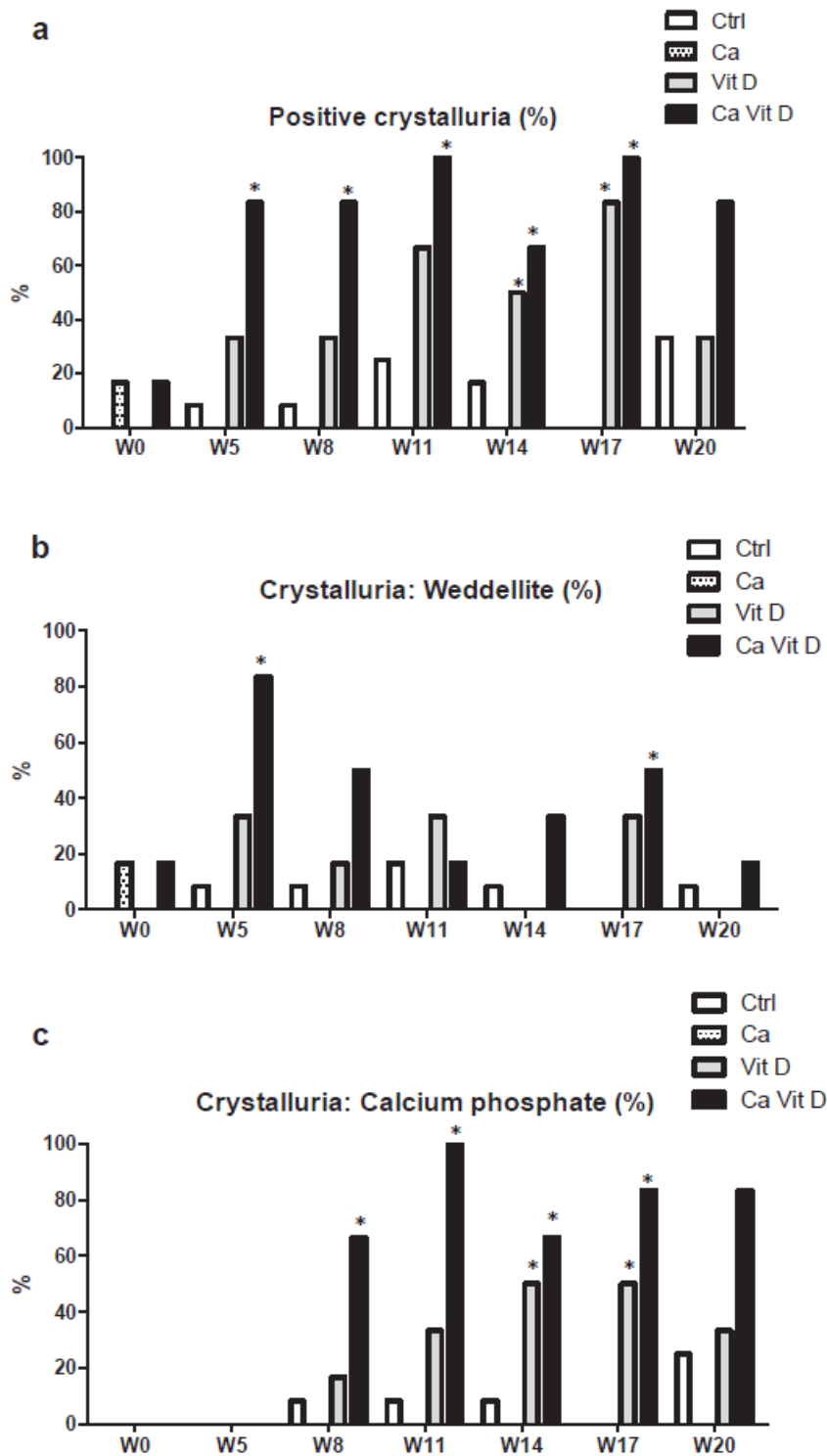
Figure 1. Evolution of crystalluria over the time. Positive crystalluria has been defined by the presence in fresh urine of calcium oxalate and/or calcium phosphate crystals (Figure 1A). Calcium oxalate crystals appeared to be more frequent during the first weeks (Figure 1B) whereas calcium phosphate crystals were abundant after 8 weeks (W8), especially in rats receiving both calcium and vitamin D (Figure 1C). Fresh urine has been analyzed before treatment (week 0-W0) and until 20 weeks (W20), before each injection of vitamin D, in the four groups: controls (Ctrl), rats receiving only calcium (Ca), vitamin D (Vit D) or calcium plus vitamin D (Ca VitD). Results are expressed as percentage (%) of positive samples.
*: $p < 0.05$ vs controls, Fisher's exact test.

2.3 Urine biological parameters

Calcium urine levels increased gradually during 6 months in rats receiving vitamin D and vitamin D plus calcium. After 8 weeks, the urinary calcium excretion was higher in the vitamin D plus calcium group than in the control group. Urinary calcium excretion increased significantly in the vitamin D group after the week 14 (Figure 2A). In addition, urinary calcium levels were higher in the vitamin D plus calcium group than in rats receiving only vitamin D, especially at 14 weeks ($p < 0.05$). We observed lower oxalate levels in the two groups of rats receiving high amounts of calcium (Figure 2B, $p < 0.05$). Urinary phosphate and magnesium levels were similar in the four groups (Figure 2C-D).
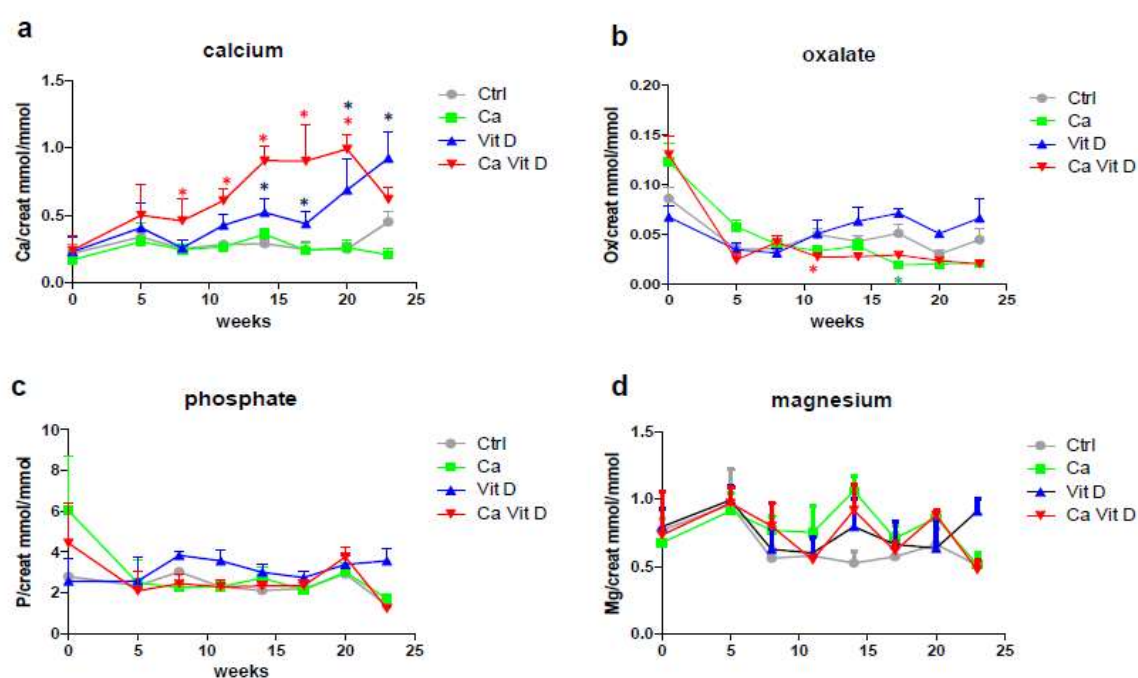

Figure 2. Calcium, oxalate and phosphate urinary excretion. Calcium urinary excretion (Ca/creat: calcium/creatinine urinary ratio) increased dramatically and early (week8-W8) in the calcium plus vitamin D group (Ca VitD), and to a lesser extent in rats receiving only vitamin D (Vit D) whereas it remained stable in rats receiving calcium (Ca) and controls (Ctrl), (Figure 2A). Oxalate urinary excretion (Ox/creat: oxalate/creatinine urinary ratio) decreased in the two groups receiving calcium when compared to controls (Figure 2B). Phosphate urinary excretion (P/creat: phosphate/creatinine urinary ratio) did not differ significantly between the four groups (Figure 2C) at any time. Magnesium urinary excretion (Mg/creat: magnesium/creatinine urinary ratio) did not differ significantly between the four groups (Figure 2D) at any time.
*: $p < 0.05$ vs controls, Mann Whitney test.

2.4 X-ray microtography (micro-CT).

Micro-CT analyses have been performed to assess kidney papillary calcifications and stones (Figure 3A-B). Rats exposed to vitamin D and calcium had many stones when compared to the other groups. In addition, intratubular calcifications have been observed. The global volume of the calcifications (including tubular calcifications and stones) has been quantified after 3D kidney reconstruction (Figure 3C-F). Rats exposed to calcium or vitamin D had a mean volume of calcifications of 0.010 mm^3 and 0.017 mm^3 respectively (p=NS vs controls: 0.003 mm^3) whereas rats exposed to both vitamin D and calcium had a mean volume of 0.121 mm^3 (p<0.05 vs controls, Figure 3G). Interestingly, most of kidney calcifications were stones, and to a lesser extent intratubular calcifications (Figures 3G-H).
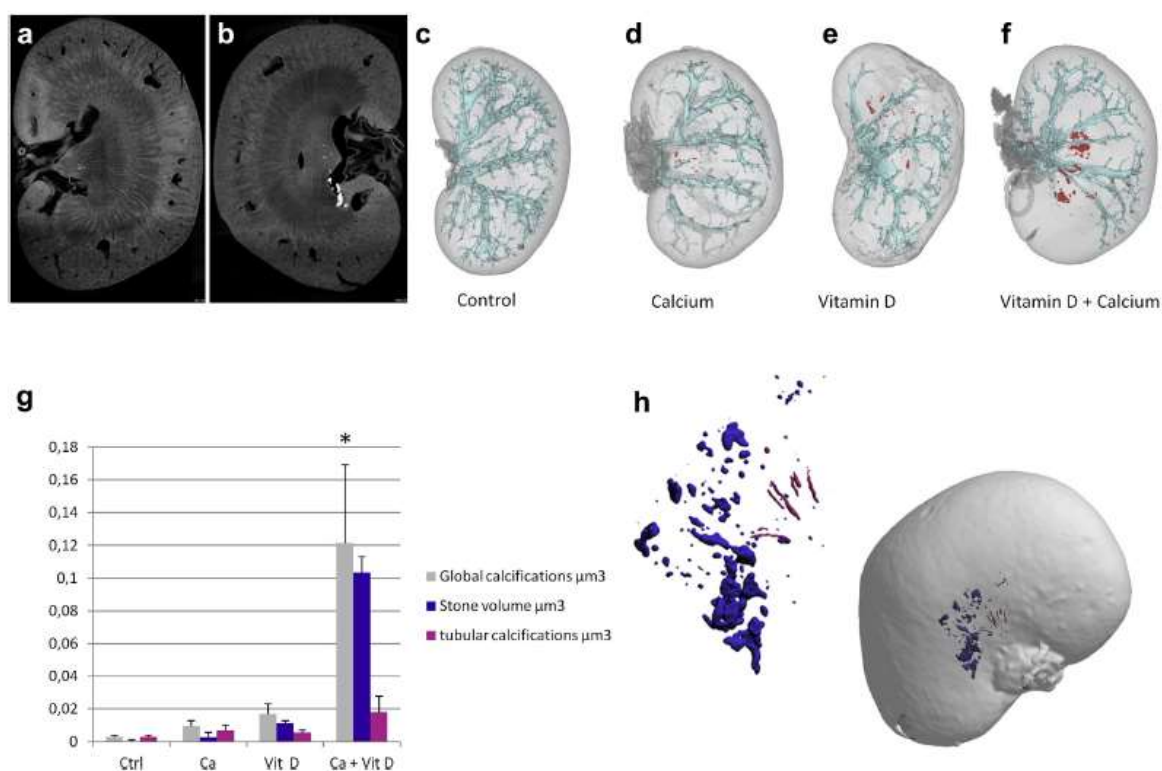

Figure 3. X-ray microtography (micro-CT). Micro-CT analyses allowed the identification of kidney stones (Figure 3A-B). Rats exposed to vitamin D and calcium had many stones when compared to the other groups. The global volume of the calcifications (in red) has been quantified after 3D kidney reconstruction (Figure 3C-F). Only rats exposed to both vitamin D and calcium had a significantly increased volume of calcifications (*p<0.05 vs controls, Mann Whitney test, Figure 3G). Most of kidney calcifications were stones (blue), and to a lesser extent tubular calcifications (pink), in rats exposed to both calcium and vitamin D (Figures 3G-H).

2.5 Histopathological analyses

Von Kossa and Yasue staining revealed the presence of calcifications in some kidney tubules and mainly stones in the urinary space (Figure 4A-E).
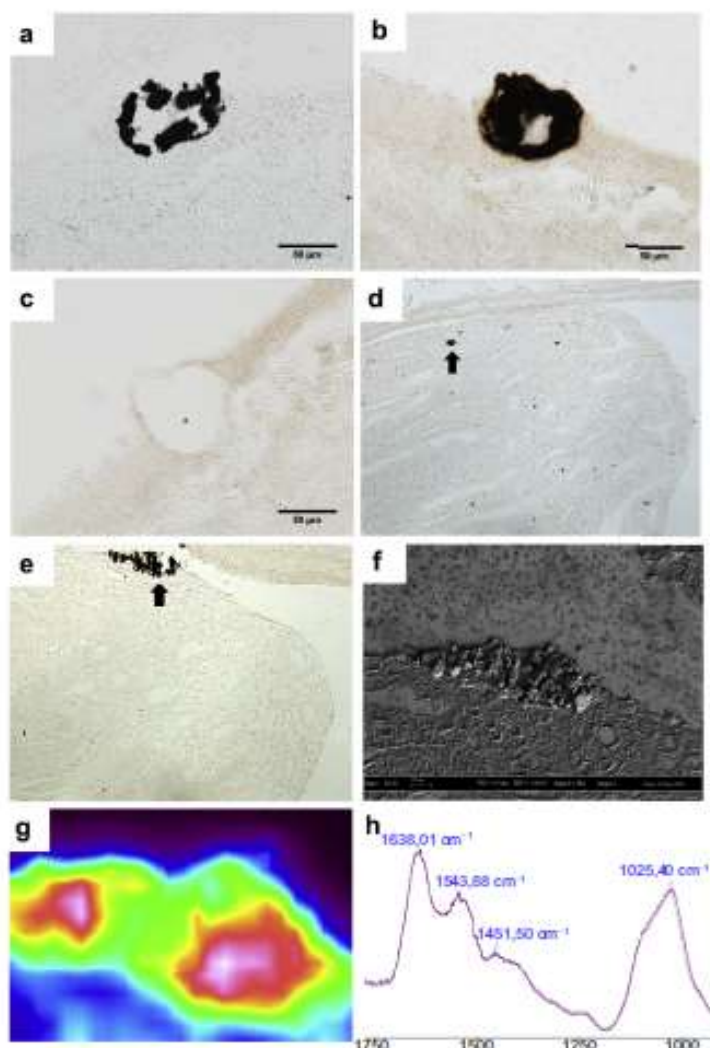

Figure 4. Characterization of kidney stones and tubular calcifications. Most of kidney stones revealed by micro-CT were removed during the immunohistochemical procedures but Von Kossa and Yasue staining revealed remnant stones in close contact to urothelium in kidneys of rats exposed to both calcium and vitamin D (Figures 4A-B, magnification x600, and 4E, magnification x100). Figures 4A, 4B and 4C correspond to serial sections stained by Von Kossa, Yasue and Yasue + acetic acid, respectively. Acetic acid removed calcifications, suggesting the presence of calcium phosphate rather than calcium oxalate. Some of these stones appeared to be surrounded by urothelial cells or fibrous caps (Figure 4B and 4C, magnification x600). Tubular calcifications were observed in the renal papilla in rats exposed to calcium and vitamin D (Arrow, Figure 4D, Magnification x100, Yasue staining) but there was no evidence of Randall's plaque formation or nephrocalcinosis at the tip of the papilla (Figures 4D and 4E, Magnification x100). Scanning electron microscopy revealed that kidney stones were round-shaped structures (Figure 4F) made of apatite and to a lower extent amorphous carbonated calcium phosphate as evidenced by FTIR spectra (Figures 4G and 4H). Figure 4G illustrates the apatite distribution (red) in kidney stones (infrared spectroscopy-based cartography) and Figure 4H is a typical spectrum of carbonated apatite and ACP mixed to tissue proteins.

The comparison with micro-CT reconstructions evidenced that most of kidney stones were removed during the preparation of kidney sections. Nevertheless, some stone fragments were still present in the urinary space, stranded in close contact with the papilla. The addition of acetic acid during Yasue staining procedure removed tubular calcifications and stones, suggesting that they were made of calcium phosphate rather than calcium oxalate (figures 4B and 4C). In some cases, adherent stones surrounded by urothelial cells or fibrous caps have been observed (Figure 4C). Interestingly, we observed only very sparse interstitial microcalcifications at the tip of renal papillae in some rats receiving both calcium and vitamin D but no structure mimicking Randall's plaque as observed in humans, and no nephrocalcinosis (Figures 4D-4E).

2.6 Field Emission-Scanning Electron Microscopy (FE-SEM) and µFourier Transform InfraRed (µFTIR) spectroscopy

FE-SEM confirmed the topography and the crystalline nature of the Von Kossa-positive deposits, especially in the urinary space (Figure 4F). To go further in the characterization of the crystalline phases, we performed µFTIR spectroscopy with an imaging system. The analysis of the absorption spectrum and its second derivative revealed some features specific for the presence of different absorption bands of the apatite $[Ca_5(PO_4)_3(OH)]$, especially the v_3 P-O stretching vibration mode measured at 1035-1045 cm^{-1} (Figures 4G-H). Of notice, carbonate ions were detected together with apatite by their v_3 C-O stretching vibration mode around 1420 cm^{-1} and the v_2 C-O bending mode at 875 cm^{-1}. The presence of amorphous calcium phosphate (ACP), revealed by the partial disappearance of the shoulder of the v_3 P-O absorption band of apatite, has been observed in most of stones and intratubular calcifications (Figures 4G-H). By contrast, the presence of calcium oxalate has not been evidenced. Of notice, the composition of intratubular calcifications and kidney stones was similar (carbonated apatite and ACP).

3. Discussion

The long term administration of calcium and vitamin D in rat increases progressively urinary calcium concentration and the onset of urolithiasis. Calcium or vitamin D supplementation alone is not sufficient to promote significant kidney stone growth. These results raise concerns about the combined prescription of calcium and vitamin D supplementation, a frequent setting in postmenopausal women.

For a long time, vitamin D has been prescribed to prevent rickets and bone mass loss (16). More recently, a craze for vitamin D has been generated by the observation of an association between low serum concentrations of 25(OH)D and a variety of non-skeletal diseases, including cancer, cognitive decline, hypertension, mood disorders, multiple sclerosis, cardiovascular diseases or metabolic syndrome (17). For these reasons, it has been recommended that 25(OH)D serum levels should stand above 30 ng/mL, a level that is not achieved in the absence of supplementation by a large part of the population (18). Nevertheless, the systematic review of the intervention studies involving vitamin D supplementation failed to evidence any effect of vitamin D supplementation on disease occurrence (17). The discrepancy between observational and intervention studies suggests that low 25(OH)D serum level is a marker but not a cause of disease. More surprising, recent studies failed to evidence a role of vitamin D supplementation in the reduction of bone mass loss (19). On the one hand, calcitriol and VDR stimulation may protect against bone mass loss

by enhancing calcium digestive absorption. On the other hand, it has been evidenced that VDR activation may result in increased bone resorption (20,21). As a consequence, vitamin D administration is accompanied by increased calcium intakes to avoid bone demineralization.

Calcium prescription is recommended in postmenopausal women to prevent bone mass loss (22). It has been shown that calcium intakes within normal ranges, unlike salt and proteins, does not promote hypercalciuria (23,24). Higher dietary calcium has even been associated to a lower risk of kidney stones (25). This might be explained by the intestinal formation of calcium-oxalate complexes reducing oxalate absorption and thereby calcium oxalate urinary supersaturation. We actually observed in our murine model a decrease of oxalate urinary excretion in groups receiving high amounts of calcium. Therefore, normal calcium intakes are highly recommended in kidney stone formers since this population is particularly at risk of bone demineralization (26,27). According to Thacher et al., normal calcium intakes seem to be more important than vitamin D supplementation in Nigerian Children to prevent nutritional rickets (28).

Although dietary calcium (at least in normal ranges) is not a risk for kidney stone formation, the results of the WHI study in a large population raise questions about the potentially harmful role of calcium and vitamin D combination. The "calcium plus vitamin D" murine model highlights the synergistic role of this association.
Interestingly, this model shares similarities with the main murine model of kidney stone disease, the GHS rat. Actually, GHS rats develop stones in the urinary space whereas most of murine models developed in mice generate intratubular calcifications and crystalline nephropathies rather than stones mimicking human urolithiasis. Bushinsky et al. have shown that stone formation in GHS rats results from increased intestinal calcium absorption and bone resorption, attributed to VDR and response to calcitriol (6,8,29). Dietary calcium has a major influence on kidney stone formation in GHS rats (30). GHS rats also form calcium phosphate stones under standard diet (31). The model described herein might also be useful to analyze the impact of vitamin D with or without calcium enriched-diet on calcium intestinal net flux and on bone turn-over in further studies. The impact of lower doses of vitamin D in the GHS rat model also deserves further studies.

Of notice, stones were mainly made of calcium phosphate (carbonated apatite and amorphous calcium phosphate) and contained no detectable amount of calcium oxalate. This might be explained for a part by the high urinary pH of rodents (almost always above 7), promoting calcium phosphate supersaturation. On the other hand, oxalate excretion was reduced after 14 weeks in rats receiving calcium or both calcium and vitamin D. Interestingly, the prevalence of calcium phosphate stones has been reported to increase in past 2 decades and one may hypothesize that the combined administration of calcium and vitamin D in the population could promote calcium phosphate stones prevalence (32).

Interestingly, despite hypercalciuria and calcium phosphate supersaturation, we observed kidney stones and tubular calcifications but no nephrocalcinosis and only very sparse calcium phosphate deposits at the tip of the papilla in some rats exposed to calcium plus vitamin D, but no development of Randall's plaque as observed in humans (Figure 4A-B). Most of stones present in the urinary space have been removed by histological procedures but some stones, sometimes covered by urothelial cells and/or fibrous caps, have been observed. It seems likely that crystal clearance previously described in tubular cells may also be performed by urothelial cells (33). These calcifications are stones and not plaques: they predominate in the urinary space on the side of the papilla, not at the tip of the papilla, and are massive and homogeneous calcifications, not diffuse interstitial calcium phosphate deposits. One may hypothesize that rodents are protected against kidney interstitial calcifications or that 6 month exposure to hypercalciuria is too short to induce plaques.

Our study suffers from limitations. First, as most of murine models of urolithiasis, kidney stones are made of calcium phosphate but not calcium oxalate as frequently observed in humans. Second, we used very high doses of vitamin D to induce kidney stone formation: 100,000 IU/Kg intramuscularly every 3 weeks. These doses are higher than doses used in human beings but were necessary to increase urinary calcium excretion in rats. As a matter of comparison, children affected by rickets (weight 12.3 ± 3.4 Kg) are treated by injections of 600,000 IU vitamin D intramuscularly (28). Interestingly, rats received supra-physiological doses of vitamin D, and serum vitamin D levels were very high in the 2 groups receiving vitamin D, but the association of these high doses of vitamin D and calcium intakes allowed to obtain urinary calcium levels similar to those observed in humans affected by kidney stones and did not induce hypercalcemia, ruling out the hypothesis of an intoxication to vitamin D. It seems likely that rodents, at least Sprague-Dawley rats, are less sensitive to vitamin D than humans. At last, we used male rats only to avoid the influence of oestrogens in calcium intestinal absorption and it would be of interest to determine in further studies whether the combination of oestrogens and vitamin D influences calcium absorption and thereby urinary calcium levels in this model.

In conclusion, the "calcium plus vitamin D" rat model highlights the synergistic role of calcium intakes and vitamin D supplementation in kidney stone formation. Beyond the description of a murine model of calcium phosphate kidney stones, these results raise questions about the widespread prescription of vitamin D supplementation in the general population, especially in addition to calcium supplementation, in the absence of a proven benefit.

Acknowledgements

Methods

Animals. Thirty 8-weeks old Sprague-Dawley male rats were purchased from Harlan Laboratories (France) and housed for 6 months. All efforts were performed to reduce animal suffering. They were housed in similar conditions (2 rats/cage) with a 12-h dark/light cycle and fed ad libitum on standard rat chow. All rats received standard chow containing 600 IU vitamin D3/Kg and 0.73% calcium. Twelve rats ("control" group) had a free access to water containing 80 mg/l calcium. One rat in the control group died for unknown reasons at 6 months. Six rats ("vitamin D" group) received vitamin D (ergocalciferol 100.000 IU/Kg, Sterogyl 15H®) every 3 weeks during 6 months by intramuscular injection and had a free access to water containing 80 mg/l calcium. Six rats ("calcium" group) had a free access to water containing 2g/l calcium (calcium gluconate). Six rats ("calcium plus vitamin D" group) received vitamin D injection and had a free access to water containing 2g/l calcium. Calcium and vitamin D doses were chosen to induce hypercalciuria. Environmental enrichment was routinely performed. All animal procedures of the laboratory are performed in accordance with the European Union Guidelines for the Care and Use of laboratory animals and with local Institutional Animal Care and Use Committees ("comité d'éthique en experimentation Charles Darwin C2EA-05") guidelines. A specific authorization has been obtained from ministry and local ethical committee (number 04557.02).

Biological samples and Biochemistry. Urine has been collected before the first administration of vitamin D and every 3 weeks from week 5 to week 23, before each vitamin D injection. Urine has been collected during 24 hours in metabolic cages with free access to water (enriched or not in calcium according to the group). Blood (1 mL) has been collected before the first injection of vitamin D and at the time of the sacrifice, 10 days after the last injection of vitamin D. The following parameters have been measured in urine: diuresis volume, calcium, magnesium, phosphate, creatinine, urea, oxalate. The blood samples have been analyzed for total calcium, phosphate, magnesium, creatinine, urea and vitamin D (at 6 months).

Serum and urinary creatinine and urea levels have been analyzed by enzymatic methods and by the Jaffe method on a Konelab 20 analyzer from Thermo Fisher Scientific. Calcium and magnesium serum and urinary levels were measured with the Perkin-Elmer 3300 atomic absorption spectrometer. Vitamin D serum levels have been measured by the IDS-iSYS 25-Hydroxy Vitamin D immunoassay. Oxalate urinary levels have been measured by ionic chromatography (Dionex ICS-3000). Fresh urine has been collected after spontaneous voiding to perform crystalluria every 3 weeks from week 5 to week 20, before vitamin D injection. The number and type of crystals has been analyzed by trained technicians (34).

Micro-Computed Tomography and 3D modelling

Left kidneys were fixed in formaldehyde, embedded in paraffin and subjected to X-ray Computed Tomographic (CT) imaging at the AST-RX platform of the Museum National d'Histoire Naturelle (MNHN), using a GE Sensing and Inspection Technologies phoenix|x-ray v|tome|x L240-180 CT scanner. We used the nanofocus XR source to obtain a 10 μm resolution scale. Data were reconstructed using datos|x® reconstruction software (Phoenix|X-ray, release 2.0) and then exported into a 16 bits TIFF image stack of virtual slices. We used Mimics Innovation suite 16.0® for the analysis, quantification of calcification volume (stones and tubular calcifications) and 3D modelling of stones, tubular calcifications and kidney vessels.

The term "stone" refers to concretions out of the papilla, in the urinary space. The term "tubular calcification" refers to concretions present within papilla in tubular structures. The term "Randall's plaque" refers to interstitial calcium phosphate deposits appearing around loops of Henle or vasa recta in kidney interstitial tissue.

Histology and Von Kossa/Yasue staining

Kidney tissues were fixed in AFA and formalin and embedded in paraffin. Four-μm tissue sections have been performed and stained by Von Kossa and Yasue procedure to reveal calcifications.

Field Emission-Scanning Electron Microscopy (FE-SEM)

Four-μm tissue sections were investigated with a Zeiss SUPRA55-VP Field Emission scanning Electron Microscope (FE-SEM). Measurements were performed at low voltage (1.4KeV) and without the usual deposits of carbon at the surface of the sample.

μFTIR spectroscopy

Microcalcification phases were characterized using μFourier Transform InfraRed spectrometry. Four micrometer tissue sections were deposited on low emission microscope slides (MirrIR, Keveley Technologies, Tienta Sciences, Indianapolis). FT-IR hyperspectral images were recorded with a Spectrum spotlight 400 FT-IR imaging system (Perkin Elmer Life Sciences, France), with a spatial resolution of 6.25 μm and a spectral resolution of 8 cm⁻

[1]. The spectra were recorded in the 4000-700 cm^{-1} mid-InfraRed range. Each spectral image, covering a substantial part of the tissue, consisted of about 30,000 spectra.

Statistical analyses. Data are expressed as percentages or mean± SEM. Mann-Whitney and Fisher's exact tests were used to compare the different groups using SAS and Statview softwares. The level of significance was set to < 0.05.

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