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Low *Oxalobacter Formigenes* Colonization is Associated with Reduced Bone Mineral Density in Urinary Stone Forming Patients

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Key Words
Urolithiasis • Bone mineral density • *Oxalobacter formigenes* • Oxalate degradation

Abstract

Introduction: Lower bone mineral density (BMD) and reduced *Oxalobacter formigenes* colonization are common findings in urolithiasis patients. But none of the studies conducted investigated the relationship between decreased bone mineral density and reduced Oxalobacter colonization. Here we evaluated the relation between BMD and *O. formigenes* colonization in urolithiasis patients. ***Materials and Methods:*** 50 stone formers (48.9 ± 11.9 years) and 50 control (47.2 ± 13.4 years) adult male subjects were included in the study. Alterations in *O. formigenes* colonization were determined as absolute *O. formigenes* count from fecal samples by real time polymerase chain reaction using species specific primers. BMD was evaluated from t- and z- scores calculated by using dual energy absorptiometry in the total femoral neck and lumbar spine (L2–L4). ***Results:*** Low BMD was observed in 18 (36%) urinary stone forming patients and in 7 (14%) control subjects in the lumbar area ($p < 0.05$). The mean *O. formigenes* count in stone formers and control subjects were 19,257 (5,791± 1,117.93) and 143,850 (2,815,725 ± 3,946,044.7) ($p < 0.05$) respectively. We observed a correlation between decreased lumbar BMD and *O. formigenes* colonization and testosterone levels in stone formers. Our results indicated that diminished *O. formigenes* colonization in the gut of urinary stone forming subjects was associated with reduced BMD.

Introduction

Urolithiasis is one of the most common benign urological diseases. Epidemiological data indicate an increase in the incidence and prevalence of urolithiasis [1]. The lifetime prevalence of kidney stone disease is estimated to range between 1% and 15%. Adult males are affected 2 or 3 times more frequently than adult females, but the gender gap has been declining in recent years. Occurrence of urolithiasis is relatively rare before age 20, and peaks in the fourth and sixth decades of life. Accumulated evidence from studies showed that the prevalence

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and incidence of urinary stone disease are in direct correlation with the patient's body mass index (BMI) in both sexes [2].

Urinary calculi are composed of several elements with calcium being the most prevalent constituent. Calcium oxalate stones make up about 60% of mixed calcium oxalate stones while hydroxyapatite constitutes 20% of all urinary stones [3]. Studies show that urinary oxalate excretion is a significant factor in stone formation [4, 5]. Recent studies investigating oxalate urolithiasis mainly focused on the role of the colonic gram negative-anaerobic bacterium *Oxalobacter formigenes*. Several studies support the role of *O. formigenes* in regulation of serum oxalate levels while reporting an inverse correlation between *O. formigenes* colonization and stone formation and recurrence [6, 7]. This bacterium uses oxalate as its main carbon and energy source. As a result of this, colonization of the colon by *O. formigenes* decreases the intestinal oxalate concentrations and oxalate hypoabsorption resulting in reduced risk of calcium oxalate stone formation [8].

Several studies have shown that bone mineral density (BMD) is reduced in calcium oxalate urolithiasis [9–11]. Despite the fact that previous studies have shown that both decreased BMD and *O. formigenes* colonization are associated, the relation between *O. formigenes* colonization and BMD in urinary stone forming patients has not been investigated. Therefore we decided to explore this possible association with a real time polymerase chain reaction (PCR) based system using a species specific primer pair.

Material and Methods

Patients and Controls

Fifty male calcium oxalate urolithiasis patients and 50 non-stone forming control subjects were included in the study. Following the approval of the local ethics committee, necessary informed consent forms were obtained from all participants. All control subjects were non-stone forming adults and in good health, as evidenced by their medical history and their normal profile on a complete metabolic serum panel. Participant who used any antibiotics in the last 3 months were excluded from the study since the use of antibiotics could decrease or in some cases completely abolish *O. formigenes* colonization [12]. Since menopausal status also affects BMD, both female stone formers and controls were excluded from the study in addition to any individual with systemic or metabolic disease which may affect bone mineral density. To minimize the uncontrolled impact on testosterone levels, patients undergoing anti-androgen therapy or those who were taking testosterone preparations due to androgen deficiency and 5-α reductase inhibitors for benign prostate hyperplasia were also excluded from the study. Total testosterone levels were measured from morning samples to adjust for the circadian rhythm in testosterone release.

Urinary Calcium and Oxalate Levels

Urinary and serum calcium levels and serum testosterone levels were measured using an automated analyzer (Model 7170; Hitachi High-Technologies, Tokyo, Japan). Urinary oxalate excretion levels were determined by using a commercial enzyme kit (Sigma Diagnostics Inc., MO, USA) from urine specimens obtained via 24-hour collection. Baseline urinary calcium and oxalate levels were compared with previously reported normal values (calcium 70–180 mg/24 h, oxalate 0.08–0.49 mmol/24 h for men). *O. formigenes* counts were quantified from stool samples which were stored at -80°C *u*ntil analysis. All subjects provided at least 3 stool samples.

Quantitative Analysis of O. formigenes

Bacterial DNA was extracted from each sample at least three times using 1g stool per sample with the i-genomic Stool DNA Extraction Kit (iNtRON Biotechnology) according to the manufacturer's specifications. The exact amount of *O. formigenes* in each sample was determined by single color real time PCR with previously described species-specific primer pairs. We used Power SYBR Green Master Mix (Life Technologies) for real time PCR and all cycling conditions were modified accordingly except annealing temperatures which were previously reported by Kumar *et al.* [13]. In addition to 45 cycles of amplification, we performed a disassociation curve analysis in order to confirm amplification specificity. A single melting point of 85°Cwas considered to indicate amplicon specificity. Primers were checked for specificity using Primer BLAST [14] and were shown to be specific for the Oxc gene of *O. formigenes*. Each reaction was run against a set of dilutions containing pure *O. formigenes* DNA (1 ng DNA = 4.82×10^5 cells) in order to obtain a standard curve. The lower limit of detection was determined as 5×10^3. When a sample yielded a quantity at the lower limit we performed conventional multiplex PCR both with the aforementioned primer pair and a universal bacterial primer pair. Universal primers were used to check if the absence of an amplicon was due to an unsuccessful PCR or to the actual absence/scarcity of *O. formigenes.* Multiplex reactions were run on agarose gels and the existence of a single band of expected size for universal primers indicated the success of the reaction and absence of bacteria. None of the multiplex reactions indicated an unsuccessful PCR.

Determination of Bone Mineral Density

BMD (g/cm^2), bone area (cm^2), and bone mineral content (g), were measured for the femur neck, and total lumbar vertebra (L2–L4) using dual-energy X-ray absorptiometry (DEXA, Hologic Inc, Waltham, QDR Elite W 4500) in both groups. Obtained data were corrected for age, height, and the BMI. These measurements were used to calculate T and Z-scores. The Z score was obtained by comparing a subject's bone density to those with the same gender and age, while the T score was obtained by comparing the bone density to 30 year old subjects with the same ethnicity and gender. The obtained values were used to express the decrease in BMD. All scores were evaluated according to the classification criteria set by the World Health Organization and expressed as the mean standard deviation [15].

Table 1. Comparison of clinical findings and other characteristics in patients and controls

Characteristics	Patients	Controls	p[1]
Age (year)	48.9 ± 11.9	47.2 ± 13.4	0.066
BMI (kg/m^2)	26.7 ± 4.5	26.1 ± 3.3	0.47
Low total testosterone[2]	18 (36%)	7 (14%)	0.008
Urine calcium (mg/24h)	267.2 ± 103,8	229.4 ± 104.5	0.073
Urine citrate (mg/24h)	260.1 ± 121.2	533.4 ± 263.9	< 0.001
Urine oxalate (mg/24h)	39.9 ± 16.4	29.5 ± 15.8	0.002
Lumbar T score < -1	19 (38%)	8 (16%)	0.013
Femoral T score < -1	10 (20%)	6 (12%)	0.275
Median OF count	19,257	143,850	< 0.001

OF = Oxalobacter formigenes; BMI = body mass index. [1]p values were separately calculated for comparison of each parameter. [2] A total testosterone concentration of < 285ng/ml is considered low.

Statistics

We used the Pearson Chi-square test for statistical analysis of lumbar and femoral BDM values and the Independent Samples T-test for the evaluation of other measurements except *O. formigenes* counts. Since the Shapiro-Wilk test showed that the number of *O. formigenes* counts was not normally distributed among patients and healthy controls ($p < 0.05$), the Mann-Whitney U test was used to assess significant differences in *O. formigenes* counts among subjects. Linear regression was used to determine the association between BDM in the lumbar region and age, BMI, testosterone levels, and *O. formigenes* counts. All statistical tests were two-tailed, and statistical significance was defined as $p < 0.05$. All analyses were conducted using SPSS version 15.0 (SPSS Inc., Chicago, Illinois, USA).

Table 2. BMD in the lumbar region is significantly correlated with testosterone levels and *O. formigenes* colonization in urinary stone forming patients

Lumbar BMD	r	p
Age (years)	0.07	0.625
BMI (kg/m^2)	0.17	0.243
Testosterone (ng/ml)	0.34	0.011
O. formigenes count	0.32	0.02

BMD = Bone mineral density; BMI = body mass index.

Results

Median *O. formigenes* counts in patients and healthy subjects were 19,257 (mean 5,791 ± 111.93) and 143,850 (mean 2,815,725 ± 3,946,044.7) respectively. Our results indicated a strong association between the number of *O. formigenes* counts and calcium oxalate kidney stone formation (Mann-Whitney U = 661, n1 = n2 = 50, $p < 0.001$). Urinary calcium levels were higher in patients but the difference was not statistically significant. Serum testosterone and urinary citrate concentrations were lower in patients when compared with control subjects while urinary oxalate levels were higher in patients. The differences in serum testosterone levels, and oxalate and citrate concentrations were found to be statistically significant. BMD measurements in the lumbar region yielded significantly lower values in patients ($p < 0.05$). BMD measurements in the femoral neck area also yielded lower values but the difference was not statistically significant ($p > 0.05$). We observed lower *O. formigenes* counts in stone formers in comparison to non-stone formers (table 1).

We also evaluated the association between BMD, age, BMI, testosterone levels, and *O. formigenes* counts using linear regression. In urinary stone forming patients, we observed a significant correlation between lower BMD and testosterone levels ($p = 0.011$, Beta = 0.34) and decreased *O. formigenes* counts ($p = 0.02$, Beta = 0.32). In addition, we also observed a negative correlation between BMD and age but this correlation was not statistically significant (table 2).

When the same comparison was performed for BMD at the femoral neck region both for healthy subjects and stone formers no significant association was detected. Upon observing a significant difference in testosterone

Table 3. Comparison of *O. formigenes* counts and total testosterone levels

	Total test < 285ng/ml	Total test ≥ 285 ng/ml	p
Urolithiasis (+)			0.025
median OF	5,510	28,610	
mean OF ± SD	110,818.9 ± 349,991	215,627.4 ± 460,553.9	0.624
Urolithiasis (-)			
median OF	163,920	137,400	
mean OF ± SD	961,760 ± 2,122,535	1,037,891 ± 2,352,958	

OF = *Oxalobacter formigenes;* SD = standard deviation.

levels we categorized patients into 2 groups as the low testosterone (< 285 ng/ml) and normal testosterone (285 ng/ml) groups and compared *O. formigenes* counts. This analysis showed a significant association between decreased *O. formigenes* colonization and low testosterone (table 3).

Discussion

Urinary oxalate excretion has a crucial role in the formation of calcium oxalate stones. Even normal concentrations of urinary oxalate have been shown to affect calcium oxalate stone formation in terms of recurrence and severity [7]. *O. formigenes* colonization has been suggested to have an impact on calcium oxalate urolithiasis since this bacterium uses oxalate as its main energy source. Batislam *et al.* [16] reported that *O. formigenes* colonization is significantly lower in stone formers and subjects with significantly diminished colonization have higher rates for the coexistence of hyperoxaluria and hypercalciuria. Other studies corroborated these results, reporting reduced urinary oxalate concentrations in calcium oxalate stone forming patients colonized with *O. formigenes* in comparison to non-colonized stone formers [17]. Our results concerning *O. formigenes* colonization are in accordance with previous findings. Urolithiasis not only affects the urinary system, but also other systems including the skeletal system. Stone formers have been reported to have a lower BMD than non-stone formers [9–11]. To the best of our knowledge our study is the first one to investigate the association between bone mineral density and *O. formigenes* colonization. Due to the lack of an established threshold value for *O. formigenes* counts in the gut we used absolute quantification to compare *O. formigenes* counts in both groups [8, 19]. We detected significantly higher *O. formigenes* counts in control subjects in comparison to calcium oxalate stone formers. In addition, we observed decreased *O. formigenes* colonization in patients with a lower BMD in the lumbar region than in those with a normal BMD. However we could not confirm these findings in the femoral neck region. In non-stone former males there was no significant difference in *O. formigenes* colonization regardless of the osteoporotic status which indicated that *O. formigenes* has little to no effect on BMD in non-stone forming men. The marked decrease in BDM in the patient group may be a consequence of the impaired calcium absorption due to the formation of non-absorbable calcium-oxalate complexes [20]. Such complexes are more likely to occur when reduced *O. formigenes* concentrations result in higher oxalate concentrations in the gut. In addition to urinary calcium oxalate excretion, BMD is also affected by testosterone levels in men [21, 22]. Therefore we measured serum testosterone concentrations in patients and control subjects and determined significantly lower testosterone concentrations in lithogenic men. This finding implies that reduced BMD may be attributed to the decreased total testosterone levels in lithogenic men when other parameters do not show a significant difference including *O. formigenes* counts. Interestingly, previous studies investigating the role of endogenous steroids in urolithiasis have produced contradictory results. Naghii et al. [23] reported significantly higher testosterone levels in stone forming men while 2 other studies conducted by Zhao et al. [24] and Watson et al. [25] showed no correlation between testosterone levels and urolithiasis. In contrast with these studies we observed an inverse correlation between calcium-oxalate stone formation and testosterone levels. When

we investigated the relation between the *O. formigenes* concentration and low testosterone levels, we observed a marked decrease in the *O. formigenes* colonization in patients with low testosterone levels. While it is possible for plasma oxalate concentrations to have an impact on the testosterone levels via an unknown mechanism, this discrepancy is probably a result of the limitations of this study. Limitations of this study include small sample size and the lack of dietary control. The effect of diet on oxalate concentrations could have been compensated for by using a food questionnaire. In conclusion, based on our findings we suggest that *O. formigenes* may play an essential role in calcium oxalate metabolism, affecting the bone mineral density in oxalate stone formers.

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