Research Report:
In Vitro Analysis of a CaOx Model to Determine the Stone Promoting/Inhibition Properties

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Abstract

Introduction: Although urinary stone disease (USD) is a common and previously studied condition, its pathogenesis is not fully understood. The interaction of factors that facilitate or inhibit the formation and aggregation of crystals is still unknown. This project uses an *in vitro* model to analyze the influence of microbial and host candidate metabolites on CaOx stones.

Methods: Eleven pre-determined metabolites were run in two assays: crystallization and growth. Samples were prepared in the lab, observed through a widefield inverted microscope and analyzed with an imaging software. The statistical analysis was done comparing the data to a negative control (creatinine) in the case of the crystallization assay, and comparing it to a negative sample (no metabolite added) for the growth assay. Three different metabolite concentrations were used to evaluate amount-dependent effects using Pearson correlation coefficient. Bioinformatics coding was run to graph the data obtained.

Results: There was statistically significant influence in most of the metabolites in the crystallization and growth metrics, as expected. A clear role in the growth assay was seen with creatine and glucoronic acid, which were notably inhibitory of stone growth, with a significant positive correlation between metabolite concentrations. Some of the hypothesized roles of each metabolite were confirmed through these experiments. However, half of the metabolites increased one metric and decreased the other, suggesting a complex interaction between them and CaOx crystals *in vivo*.

Conclusions: Understanding the effect of the candidate metabolites on the most frequent type of urinary stones can help unmask the pathogenesis of this commonly recurrent disease. Furthermore, these results can eventually aid the development of prophylactic and therapeutic recommendations for patients with USD.

Introduction

Urinary stone disease (USD) is a common disorder worldwide[1], with prevalence that ranges from 1% to 15%[2]. Furthermore, the risk of recurrence is approximately 50% over a 10 year period[3]. Calcium oxalate (CaOx) is the most common composition of urinary stones[4], and the risk grows in individuals with increased urinary oxalate concentration[5]. Hyperoxaluria, found in 5% to 25% of stone formers[6], leads to a spectrum of diseases ranging from USD, end-stage renal disease, systemic deposition of oxalate, and even death[7]. While dietary oxalate cannot be metabolized by humans, bacteria both in the gastrointestinal and urinary tracts are known to have oxalate-degrading capabilities and have been linked to USD through potential gain and loss of function mechanisms[8].

Previous studies have shown that humans constantly produce supersaturated urine with oxalate, but only 1 in 11 individuals ultimately develop symptomatic stones[9]. Consequently, factors that facilitate the aggregation of CaOx must play a role in stone formation. This observation has triggered considerable theoretical and experimental efforts in the validation of other etiologic or aggravating factors that are involved in the stone pathogenesis.

Previous studies by Miller et al[10] found that the urinary tract microbiome differentiates patients with an active episode of USD from those with no history of USD. They hypothesize that the urinary tract microbiome interacts with the host to produce a complex matrix of biomolecules that can either promote or inhibit stone formation depending on the specific bacteria present in the urinary tract. Using a metabolomic analysis of the non-crystal component of calcium-based stones, and the urine of patients with history of USD with and without recurrence, the researchers found 14 metabolites that were significantly enriched in recurrent stone formers, and that were also highly abundant in both CaOx and calcium phosphate stones. Putative identification was given to eight of these 14 biomolecules. A previous metabolomic approach has also revealed 11 specific metabolites in the urine of healthy individuals and USD patients without recurrence, indicative of a potential role in stone formation inhibition[10] (Table 1).

Given these results, there is a need to confirm and understand the role of these metabolites and the potential mechanisms that facilitate promotion or inhibition of stones. This project aims to investigate the mechanisms of interaction of microbial and host metabolites revealed in the described studies through an *in vitro* model.

Table 1. Candidate metabolites found in previous studies by Miller et al.

Stone associated metabolites	Healthy status metabolites
3-penten-2-one (methylketone)	3-glucuronide
6-methylmercaptopurine	Androsterone glucuronide
Butanal	Androsterone sulfate
Coumestrol dimethyl ether	Creatine
Estrogen (2-hydroxyestradiol)	Estriol 3-sulfate sodium salt
Ferulate	Fumaric acid
Hexanoglycine	Glucoronic acid
N-butanoyl-l-homoserine lactone	LysoPC (1-myristoyl-sn-glycero-3-phosphocholine)
	Mevalonic acid
	Progesterone (Pregnanediol-3-glucuronide)
	Testosterone sulfate

Methods

Identified metabolites were purchased and stored under the required conditions. Two assays were conducted with each metabolite based on Kanlaya *et al*[11], to quantify different aspects of the early stages of CaOx stone formation: CaOx crystallization and crystal growth. The concentration of the reagents used in the assays were derived from the average urinary concentrations of the metabolites, normalized to creatinine concentration from the previous metabolomic studies conducted in the lab. From this point, 3 magnitudes of concentrations, 1x, 10x, and 1/10x were used to evaluate the effects of the metabolite to quantify any dose-dependent impacts. An alkaline buffer composed of 10 mM Tris + 90 mM NaCl in Milli Q water at a pH of 7.4 was used to dilute each metabolite at the different concentrations, or with a no-metabolite control.

Hydrophobic metabolites, such as coumestrol and sexual hormones, were not dissolvable in the buffer. Therefore, a few experiments with ethanol, methanol, clorophorm, and cyclodextrins were tried to dilute them. The dilution was not achieved properly, hence those metabolites were not analyzed during these assay runs.

Crystallization assay

To determine the impact of metabolites on CaOx crystallization, 3 tubes with different concentrations (10x, 1x, 1/10x) of the metabolite in the buffer were prepared. In 35x10 mm petri dishes, we prepared 4 ml experiments composed by 1/3 of 1-mM $Na_2C_2O_2$ (sodium oxalate in basic buffer), 1/3 of 10-mM $CaCl_2$ (calcium chloride in basic buffer) and 1/3 of each metabolite solution. Each assay was conducted in triplicate, resulting in 9 samples per metabolite. Solutions were incubated for 60 minutes at room temperature.

After the incubation period, samples were analyzed through a widefield inverted microscope. Each dish was observed at 40 high powered fields (HPF) and images were obtained for further study.

Using ImageJ software, we quantified the number crystals from each assay (Figure 1). Particles with less than $1um^2$ surface area were excluded from further analysis.

The statistical analysis was done using an Anova test for the impact of each metabolite's crystal number, followed by a pairwise-t-test to compare each metabolite to the negative control (no metabolite).

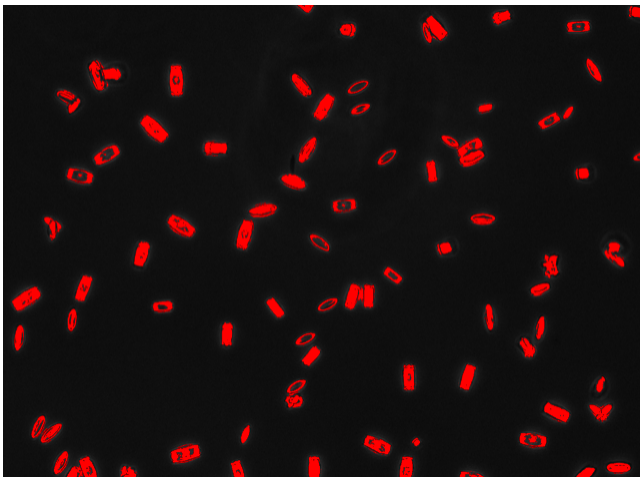

Figure 1. Selected crystals to be measured.

Growth assay

The process of sample preparation was similar to that of the crystallization assay. However, this assay required two periods of incubation. For each metabolite, we initially combined the solutions of 1-mM $Na_2C_2O_2$ and the 10-mM CaCl2 solutions to the plate. After 60 minutes of incubation at room temperature we added the metabolite,

and immediately observed (T0). The plate remained on the microscope for the second incubation period (also 60 minutes) and the same imaging process was done after this (T60).
The analysis of the images was done comparing the average sizes between T0 and T60 of the 50 largest crystals of each sample. At the same time, these results were compared to that of a negative sample (no metabolite added). Statistical analysis was done using an Anova and pairwise t-test. Pearson correlation coefficient was used to evaluate dose effect (different metabolite concentrations).

Results

A total of 11 metabolites were analyzed for the crystallization and growth assays. We observed statistically significant influence in most of the metabolites in the crystallization and growth metrics, as expected (Figure 2).
For the crystallization assay, we found that creatinine, methylmercaptopurine, lysoPC, ferulate and glucoronic acid have a promoting effect in regards to the number of crystals, however ferulate and glucoronic acid have the contrary effect when it comes to size. This behavior was not expected for lysoPC and glucoronic acid, which suggests that while they do affect crystallization, there may be some more complex interactions going on *in vivo*.
In contrast, fumarate, penten and hexanoglycine showed decreased crystal numbers, this was unexpected for penten and hexanoglycine, since both of them were predicted to promote crystal formation.
For the growth assay, creatine and glucoronic acid were notably inhibitory of stone growth, with a significant positive correlation between metabolite concentrations. Other metabolites that had a similar effect in crystal growth were ferulate, butanal and hexanoglycine, however these portrayed a negative correlation in regards to concentration. The metabolites that showed a significant effect in increasing stone growth were creatinine, lysoPC, fumarate and penten. However, as mentioned before, crystals with fumarate and penten were decreased in number, which suggests discrepancy in the influence of these metabolites, since fumarate was predicted to be present in healthy urine samples, and penten in that of stone associated urine.

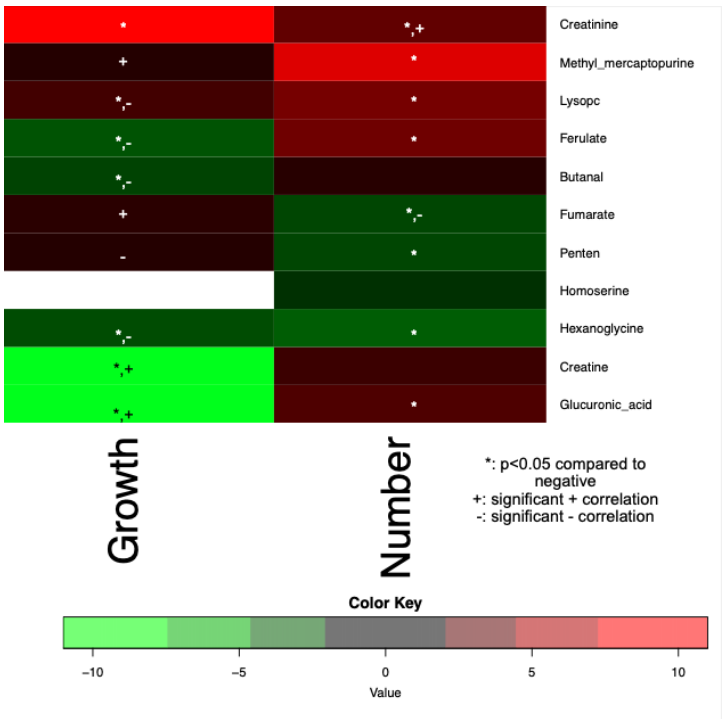

Figure 2. Metabolites that influence crystal growth. * Indicates significance compared to the negative control. +/- indicates a significant positive or negative dose-dependent effect.

Discussion

Metabolites can influence stones in different ways: crystallization, growth and aggregation (Figure 3). Although a metabolite can increase any of these, it can also decrease other metrics at the same time. The preliminary data on this study clarifies the broad relationship by demonstrating the specific direct effects of each metabolite on CaOx crystals.

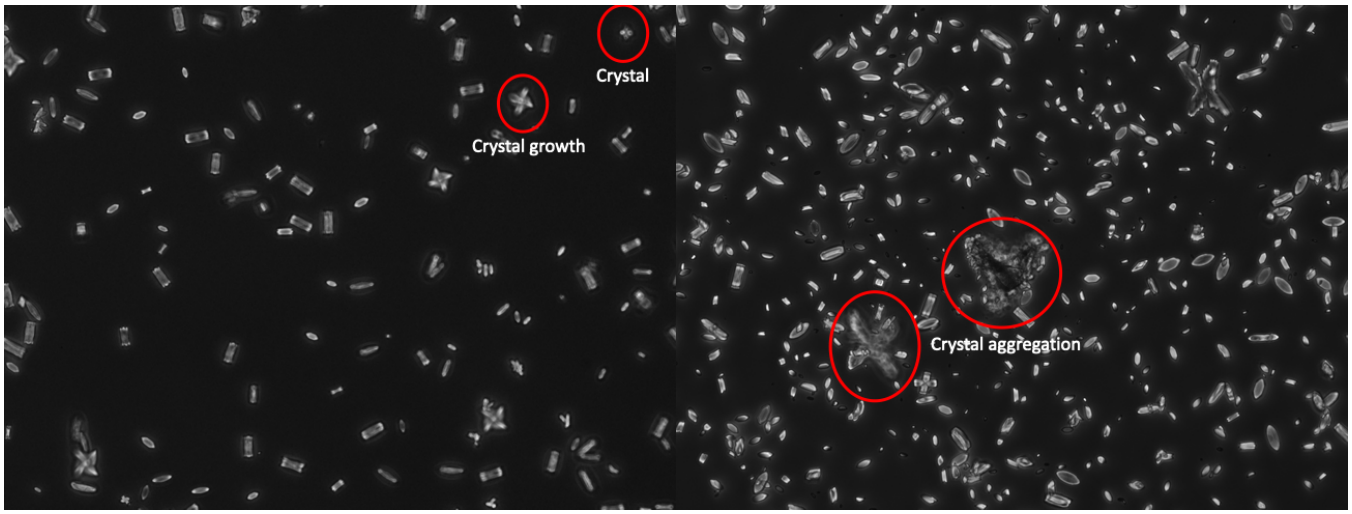

Figure 3. Crystal development.

There are not many previous studies that focus on molecular interactions of products of the urinary tract microbiome. A previous study by Kanlaya *et al*[11] studied the pathogenetic role of *E. coli* in CaOx stones. They added bacterial components to similarly composed samples to see which one promoted stone crystallization, growth and aggregation, but they did not use bacteria by-products. For that reason, this research project is innovative and the results obtained are the first step towards knowing more about host and microbiome metabolic interactions in the urinary tract.

Working with the metabolites was a challenge in itself. For some of them there was no precedent with the type of buffer we used. Solubility of some substances, like coumestrol, was not achieved. Toxic metabolites like butanal and 3-penten-2-one had to be carefully managed, 3-penten-2-one can even affect plastic composition of the tubes. Because of price and convenience, the amounts used were very small, so handling became a challenge as well. Furthermore, each solution had to be used the same day it was created, so planning and perfect execution became an important part of the project.

There was a third assay that we wanted to carry out: the aggregation assay. This one measures the amount of conglomeration of crystals in the presence of each metabolite. However, we were not able to harvest crystal precipitants during my participation, even after various experiments based on previous studies[11, 12, 13].

The most important limitation of our study is the abiotic environment. Although the behavior of each individual metabolite is relevant and can shed some light into the unknown complexity of the biomolecules presence in the urinary tract, other *in vivo* factors and interactions were not measured with the assays we performed.

Beyond these limitations, our finding may help unmask the basic effects and interactions of the candidate metabolites and USD.

Conclusions

After revealing the exact role and underlying mechanisms of stone promotion and inhibition of these compounds, a new pathway of possible disruption in the pathogenesis of urolithiasis could be explored with the aim of the development of novel therapeutics in CaOx-USD patients, especially those with recurrent episodes.

References

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