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IMPACT OF *Escherichia coli* ON URINE CITRATE AND UREASE-INDUCED CRYSTALLIZATION

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Abstract

Escherichia coli (*E. coli*) is usually not a urease producer. It is, however, often cultured in urinary phosphate containing calculi including ammonium magnesium phosphate stones. This suggests the possibility that *E. coli* might be involved in stone forming process. The effect of *E. coli* on urine citrate and urease-induced crystallization in human urine has been studied *in vitro*. *E. coli* was found to strongly reduce urine citrate (after 48 hours). In the *E. coli* inoculated samples, the urease-induced crystallization was increased. There was a strong correlation, $r = 0.8$, between the citrate decrease and the increase in calcium precipitation. The results indicate that *E. coli* and the reduced urine citrate influences urease-induced crystallization *in vitro*.

Key Words: *E. coli*, citrate, ammonium magnesium phosphate, calcium.

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Introduction

The causal relationship between urease-producing microorganisms and ammonium magnesium phosphate stones is well established. *E. coli* is, with very rare exceptions, not a urease producer [5] but it is also frequently cultured in urine from patients with urinary calculi. It occurs especially in connection with phosphate-containing stones such as stones containing ammonium magnesium phosphate and calcium phosphate, but more rarely in patients with pure calcium oxalate stones [2, 9, 11]. When *E. coli* is cultured in urine from patients with urinary tract concrements, it is generally thought to be an infection secondary to the stones. The fact that *E. coli* is cultured so often, with a frequency of 35% in upper urinary calculi [18] in patients with phosphate stones, does, however, suggest the possibility that the microorganism might be involved in the stone-forming process.

It has been known for a long time that some bacteria use citrate as the sole external source of carbon [6]. *E. coli* is not supposed to use citrate as a nutrient under standardized circumstances [10] and it is used as a negative control in the citrate utilization test (Simmons citrate test), to differentiate members of the Enterobacteriaceae [17] family. However, if urine is inoculated *in vitro* with *E. coli*, the citrate concentration is markedly reduced after 48 hours' incubation [1]. Citrate affects supersaturation in urine with respect to calcium salts [13, 15, 20] since it forms complexes with calcium ions. A low urinary citrate concentration is also a common finding in patients with calcium stone disease [8, 14, 16]. Citrate also binds magnesium ions and consequently affects the supersaturation of magnesium in urine. A question which thus comes to mind is how *E. coli* inoculation affects urease-induced precipitation and if it promotes the stone-forming process.

We have previously shown that *E. coli* influences the urease-induced crystallization [4, 7] but we have not studied if this is related to a decrease in urine citrate.

The aim of the present study was to evaluate the effects of *E. coli* inoculation on urine citrate and the influence on urease-induced crystallization.

Materials and Methods

Urine sample handling

Morning urine samples were collected from healthy volunteers. All urines were centrifuged at 1500 *g* for 30 minutes and filtered through a coarse glass microfiber filter and a 0.22 μm Millipore® filter. A check for bacteria was made with the Nitur® test (Boehringer Mannheim, GmbH, Mannheim, Germany) before and after centrifugation and filtration. The urines were kept in sterile bottles at +4°C until use. Fifteen different urine samples, 100 ml each, were used.

E. coli inoculation

The sterile urines were divided into two equal parts. One part was inoculated with 1 ml *E. coli* suspension (ATCC 25922, clinical isolate FDA strain, Seattle 1946) and the other part, without bacteria, was used as a control. The urines were kept at +37°C for 48 hours. Bacterial cultures were performed using conventional methods, and samples for citrate analysis were taken before and after the incubation.

Urease incubation

From each of the 15 pairs of *E. coli* and control urines, 15 ml was transferred, in duplicate, to glass tubes with a glass rod immersed in the urine. The tubes were incubated with 100 μl of a Jackbean urease solution (72 units·ml^{-1}) from Sigma (no. U 0376, 729,000 units per gram) for 4 hours at +37°C. pH was measured before and after the incubation. After 4 hours, the precipitation of calcium, magnesium and phosphate on glass rods was measured. At that time, 5 ml of the urine was also filtered through a 0.22 μm Millipore filter. After 24 hours, the remaining urine was filtered in the same way. The precipitate on the filters was weighed when dry and the amount of calcium, magnesium and phosphate was measured.

Analytical methods

The pH was measured by a combination electrode (VIT-90, Radiometer®, Copenhagen). Calcium and magnesium were analyzed by atomic absorption spectrophotometry [19] and phosphate was determined by a colorimetric method [12]. The determinations of citrate were done by an enzymatic method described by Tompkins and Toffaletti [21]. The urease-induced precipitation was analyzed after 4 and 24 hours by means of an Olympus BH-2 polarization microscope with a magnification of 100 and 200 times.

Statistical methods

Means, standard deviations and coefficients of correlation (r) were calculated using conventional methods. Wilcoxon's signed rank test was used to calculate the significance of differences between paired observations.

Results

The mean concentration of citrate was 1.10 ± 0.80 mmol·l^{-1} before and 0.15 ± 0.26 mmol·l^{-1} after inoculation with *E. coli* and incubation for 48 hours (Figure 1). In 10 samples, the citrate concentration was reduced to 0; in four, it was between 0.2 and 0.5 mmol·l^{-1}; and in one, there was no change at all. The mean difference was significant ($p = 0.0007$). In the controls, the mean concentration of citrate was unaltered (1.13 ± 0.84 and 1.16 ± 0.86 mmol·l^{-1} before and after, respectively, Figure 1).

The Nitur® test was negative in all urines both before and after sterile filtration.

The number of bacteria was approximately 10^6 bacteria per ml^{-1} before the incubation and 10^7 per ml^{-1} after 48 hours in the *E. coli* inoculated urine samples. There was no contamination in the control samples.

Urine pH did not change during the 48 hours incubation in the *E. coli* or the control urines (pH = 5.80), but the urease-induced pH increase was significantly lower in the *E. coli* samples compared with the controls (pH = 8.72 and 8.82, respectively, Wilcoxon's signed rank test, $p = 0.03$).

Significantly less phosphate, calcium and magnesium had precipitated on the glass rods in the samples inoculated with *E. coli* (Table 1). The amounts of calcium, magnesium and phosphate precipitated in the solution and collected on filters were, on the other hand, significantly greater in the *E. coli* urines after both 4 and 24 hours (Table 1).

The weight of the precipitate in the solution and collected on the filters was also significantly greater after both 4 and 24 hours in the *E. coli* inoculated samples compared to the controls (Table 1).

The microscopic examination of the precipitate after 4 hours revealed that there were more and larger crystals and more crystal aggregates in the *E. coli* urines than in the controls (results not shown). After 24 hours of urease incubation, more and larger (mm-sized) crystals and crystal aggregates were observed than after 4 hours. There was, however, no detectable difference between control and *E. coli* urines as observed by microscopy at this time.

There was a strong correlation ($r = 0.8$, $p = 0.01$) between the decrease in citrate and the increase in calcium precipitation in the *E. coli* inoculated urine samples. The correlation coefficients between the citrate decrease and the phosphate and magnesium precipitations were 0.42 and 0.12 respectively (not significant).

Table 1. The mean urease-induced precipitation of phosphate, calcium and magnesium on the glass rods and on the filter (the precipitation in the solution collected on the filter) after 4 and 24 hours in control samples and *E. coli* inoculated samples, and the weight of the total precipitation on the filters after 4 and 24 hours.

	Rod, 4 hours (mg·rod^{-1})		Filter, 4 hours (mg·filter^{-1})		Filter, 4 hours (mg·filter^{-1})	
	Control	**E.coli**	**Control**	**E.coli**	**Control**	**E.coli**
Phosphate	0.18	0.12**	1.28	1.42**	1.91	2.11*
Calcium	0.06	0.04***	0.31	0.34*	0.45	0.51**
Magnesium	0.013	0.009**	0.109	0.131*	0.308	0.360*
Weight			7.54	8.28**	4.68	5.55*

Wilcoxon's signed rank test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

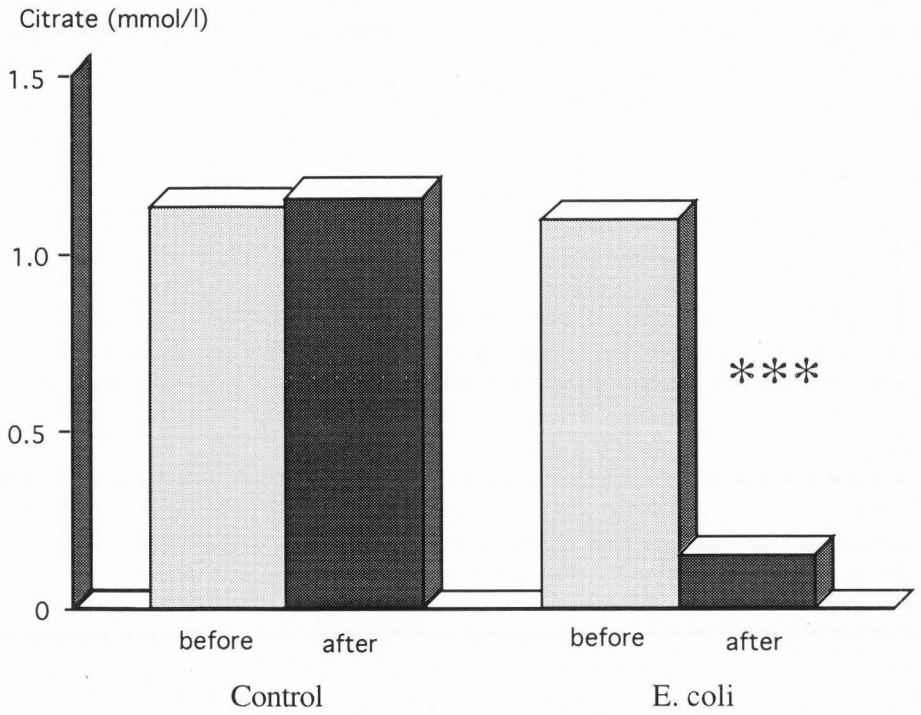

Figure 1. The mean citrate concentration in controls and *E. coli* inoculated urines before and after incubation for 48 hours (Wilcoxon's signed rank test $p = 0.0007$).

Discussion

E. coli inoculation and incubation for 48 hours gave a strong reduction of urine citrate in almost all tested urines. According to the literature, *E. coli* does not use citrate as a nutrient under standardized circumstances [10] but it has been shown to decrease the citrate concentration in urine [1]. The cause of the decrease in citrate noted in this series of experiments may be that its ordinary carbon source is lacking in most urines and that citrate is used as such. The effect of *E. coli* varied rather markedly between urines and it appears as if an incubation of more than 24 hours is needed for the citrate depletion to be detectable in urines [4]. Normally *in vivo*, the urine passes the urinary system more rapidly. Only under special circumstances, for example, obstruction in the urinary tract, could this citrate decrease be of importance.

E. coli have both a respiratory and a fermentative type of metabolism. In the fermentation of some carbohydrates [10], e.g., D-Glucose, *E. coli* produces acid and gas as waste products. The acid production may explain why the urease-induced pH increase was lower in the *E. coli* urines compared with the controls. Previous studies have shown that *E. coli* inhibits urease and the reduced urease-induced pH-increase in the *E. coli* urines may also be caused by this mechanism [7].

The urease-induced precipitation was larger in the

E. coli urines. Urease gives a precipitation of both ammonium magnesium phosphate and calcium phosphate [22]. The precipitation of both salts was increased but only the increase in calcium precipitation was strongly related to the citrate reduction. Citrate also has a stronger tendency to bind calcium than magnesium.

The precipitation on the rods immersed in the urines was reduced in the *E. coli* urines, which has also been observed in a previous study [4]. *E. coli* may act as nuclei for precipitation which compete with the rod surface. This is confirmed by the microscopic analysis of the precipitate which showed more and larger crystals in the *E. coli* urines than in the controls. This might explain why the rod precipitation was reduced when *E. coli* was present during the urease-induced precipitation. Previous studies have shown that when *E. coli* is filtered off before the urease incubation, the precipitation on the rods is not influenced [7]. The surface polymers of *E. coli* did not increase struvite mineral growth [3]. It thus appears as if intact *E. coli* must be present to increase the precipitation.

In previous experiments [4, 7] studying the effects of *E. coli* preinoculation, the precipitation of calcium was not analyzed, and the total precipitation in the solution with *E. coli* left during the urease-induced crystallization was not measured either. We chose to leave *E. coli* in the solution during the precipitation to see if that influenced the process in a different way, which it thus did. This is also closer to the *in vivo* situation during an ongoing urinary tract infection or colonization. *In vivo*, *E. coli* could be anticipated to increase the precipitation, but, on the other hand, it appears to delay the pH increase. The summation effect of this will probably vary between urines, depending on factors like the urine buffer capacity, calcium concentration and how pronounced the *E. coli*-induced citrate depletion is.

The results from this study show that *E. coli* strongly reduces urine citrate, in certain samples to undetectable values. It further influences the precipitation of the studied salt but its relevance to the stone-forming process *in vivo* needs to be proven.

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Discussion with Reviewers

R.J.C. McLean: Would you recommend bladder irrigation with citrate or some other carbon source in stone forming patients, particularly those with infection? My rational for suggesting this is twofold: (1) the inhibitory effects of some compounds such as citrate, and (2) the formation of organic acids from microbial fermentation of carbon compounds, which can then buffer against pH elevation).

Authors: Yes we would. In the case of infection stones, it is also necessary to eradicate the infection. In fact, citrate solutions (Hemacidrin) are used to dissolve renal infection stones. The method requires percutaneous pyelostomies and may require weeks for a stone to disappear.

R.J.C. McLean: Please comment on the significance of difference in Table 1.

Authors: The significance of differences were calculated using Wilcoxon's signed rank test. The number of observations were 15 in each group. The precipitation on the rods of phosphate, calcium and magnesium were significantly smaller in the *E. coli* inoculated samples and significantly larger on the filters after both 4 and 24 hours in the *E. coli* inoculated samples.

R.J.C. McLean: The strain of *E. coli* used in this experiment should be grown on Simmons citrate media or similar diagnostic media to verify if it can indeed use citrate as a carbon source.

Authors: The strain of *E. coli* (ATCC 25922) used in this experiment (see **Materials and Methods**), is a well controlled and tested strain. This regards also citrate utilization. It does not use citrate which we, after your recommendation, have proven with Simmons citrate test.