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Increased risk of urinary tract infection associated with the use of calcium supplements

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Summary. Since ions are known to influence the interaction between cells, we undertook an examination of the effect of various ions on bacterial adherence to uroepithelial cells. While most of the ions examined had no effect or decreased bacterial adherence, calcium ions significantly increased bacterial adherence. It was demonstrated, in vitro that as the concentration of calcium was increased to levels higher than normally found in the urine, there was a significant increase in bacterial adherence. It was also found that if the diet was supplemented with calcium there was an increase in the excretion of calcium in the urine and a corresponding increase in bacterial adherence when bacteria and uroepithelial cells were incubated in this urine. It is suggested that an excretion of excess calcium in the urine may lead to increased bacterial adherence in vivo and an increased potential for urinary tract infections.

Key words: Urinary tract infection – Calcium – Adherence

Community-acquired urinary tract infections account for 6–7 million doctor's office visits in the U. S. each year, and in about 20% of the individuals the disease recurs three to six times per year [14]. Accumulated evidence suggests that bacterial adherence is an important first step in the development of urinary tract infections [3, 6]. *Escherichia coli* is the most frequently isolated pathogen from individuals with diagnosed urinary tract infection [7]. *E. coli* contain type 1 fimbriae, which mediate mannose-sensitive adherence of the bacterium to uroepithelial cells [12], and P fimbriae, which express mannose-resistance adherence [8]. Via in vitro studies it has been demonstrated that P fimbriae are usually expressed on *E. coli* that are isolated from patients with upper urinary tract infections, and type 1 fimbriae on *E. coli* isolated from patients with both upper and lower urinary tract infections [2, 4, 5]. Direct in vivo expression of pili on *E. coli* has been demonstrated from freshly isolated urine of patients with pyelonephritis and cystitis via immunofluorescence, electron microscopy and immunostaining with polyclonal antiserum [3, 6].

Since adherence involves the close interaction of two cell surfaces, any factor that may interact with these surfaces could potentially change the attraction between the two cells. In this study we examined the effect of various ions on the adherence process and found that while most of the ions examined had little or no effect on attachment, calcium strongly enhanced bacterial adherence. In particular, if the normal diet was supplemented with additional calcium, there was a corresponding increase in the excretion of calcium and urine collected after supplementation significantly increased bacterial adherence.

Material and methods

Bacteria

E. coli strain 1806, isolated from a patient with diagnosed urinary tract infection ($> 10^5$ bacterial colonies/ml), was obtained from the microbiology laboratory of Alliance City Hospital. The identity of the organism was confirmed by the use of API 20E strips (Fisher Scientific), and the organism was shown to express both type 1 and P fimbriae [11]. For long term storage the bacteria were transferred to agar talls of brain heart infusion, grown at 37°C for 18 h, and then stored at 2–6°C.

Exfoliated epithelial cells

Exfoliated epithelial cells were obtained from three healthy female volunteers with no history of urinary tract infection. Epithelial cells, from freshly voided midstream specimens, were harvested by centrifugation of a 30 ml sample at 1,000 × g for 10 min and then washed once with 0.01 M phosphate buffered saline (PBS), pH 7.4

Bacterial adherence assay

Bacterial adherence was determined as previously described [11, 13]. One ml samples of bacteria containing 10^9 epithelial cells were combined and incubated in a shaking water bath at 50 oscillations/min for 30 min. Following incubation, a one ml sample was removed

Table 1. Adherence of *E. coli* to exfoliated uroepithelial cells after incubation in PBS or urine supplemented with increasing levels of calcium

Concentration of calcium in the incubation medium	Incubation medium	Mean bacteria per cell
0	PBS	24.26
1 mM	PBS	23.04
5 mM	PBS	32.33*
10 mM	PBS	33.71*
20 mM	PBS	48.14*
0	Urine	19.20
1 mM	Urine	18.04
5 mM	Urine	39.50*
10 mM	Urine	35.90*
20 mM	Urine	42.14*

* Significantly different from the control with no calcium added to the incubation medium ($P < 0.05$)

Table 2. Adherence of *E. coli* to exfoliated uroepithelial cells after incubation of the *E. coli* or epithelial cells in PBS with increasing concentrations of calcium

Concentration of calcium in the incubation medium	Cell type	Mean bacteria per cell
0	*E. coli*/epithelial	7.79
5 mM	*E. coli*	4.96
10 mM	*E. coli*	27.66*
20 mM	*E. coli*	35.78*
5 mM	Epithelial	14.16
10 mM	Epithelial	17.66*
20 mM	Epithelial	20.96*

* Significantly different from the control with no calcium added to the incubation medium ($P < 0.01$)

and filtered through a 8 μm polycarbonate membrane and washed three times with 10 ml of PBS to remove nonadherent bacteria. The filters were placed on a glass slide, allowed to dry, and then removed. The adhering cells were Gram-stained and a total of 50 epithelial cells were scored for each sample. Statistical comparisons between means were done by analysis of variance (ANOVA). For the incubation studies, 1 ml samples of the bacteria or the epithelial cells were centrifuged, resuspended in the test medium, and incubated at 37°C for 10 min with shaking. The samples were centrifuged for 10 min at 1,000 × g to pellet the cells and then used in the adherence assay.

Dietary studies

A healthy female with no history of urinary tract infection was placed on a nondairy diet of the same foods for three consecutive days. On the 2nd day, first morning urine was discarded and then urine was collected continuously until bedtime. This urine was labeled pre-day no calcium. The following morning, the first morning urine was collected and labeled pre-night no calcium. A total of 1,500 mg of calcium carbonate was taken, 500 mg with each meal, and the urine collected until bed time and labeled day plus calcium. The following morning, the first morning urine was collected and labeled postday plus calcium. Adherence studies were performed via the incubation method using the pooled urine. The urinary calcium was measured via the Sigma Diagnostic Kit number 587.

A second study was performed in which the same subject ingested 500 mg of calcium carbonate and urine samples were taken at 2, 4 and 8 hours and urinary calcium was measured.

In a third study, a 24-hour urine sample was collected and pooled to serve as the control. On day two, 500 mg of calcium carbonate was taken and urine was collected after 2 h. On day three, 500 mg of calcium carbonate and ten ounces of cranberry juice cocktail were taken and urine was collected after 2 h. *E. coli* and the epithelial cells were incubated in the 3 urine samples and then tested for adherence.

Results

Adherence of E. coli to exfoliated uroepithelial cells

For each experiment in which adherence was measured, exfoliated uroepithelial cells were incubated without the addition of *E. coli* to serve as a control. The mean number of bacteria on these cells ranged from 0 to 0.06. The addition of *E. coli* to the incubation medium resulted in adherence levels that were always significantly higher ($P < 0.01$) than the control values and ranged from a mean of 8 to 35 bacteria per cell.

Effect of calcium on bacterial adherence

Addition of calcium to the incubation medium increased the level of bacterial adherence (Table 1). When *E. coli* and exfoliated uroepithelial cells were incubated in sterile PBS, the mean number of bacteria adhering to each cell was 24.26. The addition of calcium, as calcium chloride at 5,10 or 20 mM to the incubation medium, significantly increased the level of bacterial adherence respectively to 32.33, 33.71 and 48.14 mean bacteria per cell. Calcium at 1 mM had no effect on adherence (23.04 mean bacteria/cell).

Since normal urine is known to contain ionized calcium plus substances that complex calcium ions, sterile urine was also used as the incubation medium. The results are identical to those presented above for the PBS (Table 1). In the controls with no added calcium the mean bacteria/cell was 19.20. Increased adherence was observed with the addition of 5,10 and 20 mM calcium chloride (39.50, 35.90 and 42.14 mean bacteria/cell), and no increase (18.04 mean bacteria/cell) was observed when 1 mM calcium chloride was added to the sterile urine.

Adherence after incubation in media containing various concentrations of calcium chloride

In order to determine if calcium had to be continually present in the medium to increase adherence, *E. coli* or epithelial cells were incubated in a calcium-containing medium, and then shifted to another medium without added calcium to allow adherence. The cells were incubated in either sterile PBS or urine for 10 min, with or without added calcium, and then incubated in sterile urine for the adherence test. The results of incubation in sterile PBS are presented in Table 2.

Table 3. Effect of incubation of *E. coli* or uroepithelial cells in 10 mM calcium, magnesium, sodium or potassium salts on bacterial adherence

Ion added	Cell type	Mean bacteria per cell
None	*E. coli*/epithelial	16.88
Calcium	*E. coli*	35.14*
	Epithelial	42.80*
Sodium	*E. coli*	18.95
	Epithelial	11.18
Potassium	*E. coli*	10.91
	Epithelial	19.08
Magnesium	*E. coli*	8.39*
	Epithelial	10.11*

* Significantly different from controls in which no salts were added to the incubation medium ($P < 0.05$)

Incubation of *E. coli* in either 10 or 20 mM calcium chloride resulted in a significant increase in bacterial adherence (27.66 and 35.78 mean bacteria/cell) as compared to controls (7.79 mean bacteria/cell) which had no added calcium. If the level of calcium was reduced to 5 mM, no increase in adherence was observed (4.96 mean bacteria/cell). A similar pattern was observed when uroepithelial cells were incubated in a calcium-containing medium. At a concentration of 10 or 20 mM calcium chloride a significant increase in adherence was observed (17.66 and 20.96 mean bacteria/cell), and no increase in adherence with 5 mM calcium chloride (14.16 mean bacteria/cell). If sterile urine was substituted for the sterile PBS as the incubation medium, the results were similar with concentrations of calcium above 10 mM increasing adherence and those 5 mM or less having no effect on adherence.

Interaction of calcium with the cell surface

Based on the previous experiments it appeared that calcium associated with both types of cells and need not be continually present to influence adherence. To determine the stability of this interaction, bacteria or epithelial cells were incubated for 10 min with 10 mM calcium chloride and then subjected to a series of washes with PBS followed by the adherence assay. After incubation in sterile PBS with 10 mM calcium chloride, the cells were pelleted and mixed with five ml of sterile PBS, pelleted again, and subjected to the adherence assay in sterile urine. A total of 0–5 washes were performed. For the controls with no calcium added to the incubation medium, the mean number of adhering bacteria was 19.22 per cell. Washing these cells a total of 4 times did not significantly decrease adherence. Incubation of either *E. coli* or epithelial cells in 10 mM calcium chloride increased adherence values significantly ($P < 0.01$) to 37.08 and 42.60 respectively. Adherence dropped significantly ($P < 0.01$) to 28.04 after 1 wash for *E. coli* and in subsequent washes the values for adherence dropped significantly ($P < 0.01$) to 27.96, 18.44 and 18.14. For washes 3 and 4, the adherence values were not significantly different ($P = 0.782$ and 0.708 respectively) from control adherence values in the absence of calcium. The pattern was slightly different for the epithelial cells. After 1 wash the mean bacteria per cell was 43.90 which is not significantly different ($P = 0.66$) from adherence values with no wash. However, after a 2nd and subsequent washes the values dropped significantly ($P < 0.01$) to 27.34, 24.98 and 17.89. After the 4th wash the adherence value was not significantly different ($P = 0.704$) from the control value incubated in the absence of added calcium.

Effect of ions other than calcium on bacterial adherence

Since it appeared from the previous experiments that the effect of calcium on bacterial adherence was related to a loose interaction between the cell surface and the calcium ion, other ions were tested to determine if they would also effect adherence. All the ions were tested as chloride salts to eliminate the possible effect of chlorine. Chlorine salts of calcium, magnesium, sodium and potassium were tested (Table 3). The concentration used was 10 mM, the lowest concentration of calcium salts that consistently gave an increase in bacterial adherence. It can be observed from the table that only incubation in calcium chloride of both the bacteria and the epithelial cells increased bacterial adherence (35.14 and 42.80 mean bacteria/cell). Controls contained 16.88 mean bacteria/cell. Salts of sodium and potassium had no effect on adherence with *E. coli* (18.95 and 10.91 mean bacteria/cell) or epithelial cells (11.18 and 19.08 mean bacteria/cell). Magnesium salts decreased the level of bacterial adherence to 8.39 and 10.11 mean bacteria/cell when incubated with *E. coli* and epithelial cells respectively.

Effect of calcium and an anti-adherent agent on bacterial adherence

In an attempt to reduce the increase in adherence observed with calcium an anti-adherent agent was used. *E. coli* and epithelial cells were incubated with and without 10 mM calcium, and with cranberry juice cocktail plus 10 mM calcium. The mean number of adhering bacteria in the control sample was 29.61. With the addition of 10 mM calcium the value increased significantly ($P < 0.01$) to 47.22 and decreased significantly ($P < 0.01$) to 21.27 when cranberry juice cocktail was included in the incubation medium. When both calcium and cranberry juice were included in the incubation medium, the mean number of bacteria per cell was 22.11 which was significantly ($P < 0.01$) lower than both the control and the sample containing 10 mM calcium.

Evaluation of urine after dietary supplementation with calcium

Calcium carbonate, in the form of a commonly used antacid, was used as the source of dietary calcium. A total

Table 4. Effect of incubation of *E. coli* or epithelial cells in urine collected before and after dietary calcium supplementation

Hours before (−) or after (+) supplementation	Cell type	Mean bacteria per cell
−24	*E. coli*	15.30
−12	*E. coli*	12.67
+12	*E. coli*	23.56*
+24	*E. coli*	28.02*
−24	Epithelial	16.94
−12	Epithelial	13.23
+12	Epithelial	28.48*
+24	Epithelial	23.66*

* Significantly different from the −24 and −12 readings ($P < 0.01$)

of 1,500 mg of calcium carbonate was taken over a twelve hour period. Urine was collected and pooled over four 12-h time periods; for 2 12-h time periods prior to taking the supplement, for the 12-h period when the calcium supplement was taken and for 12 h following calcium supplementation. The calcium concentration in the four pooled samples was then measured. The pre-day 12-h sample contained 33.7 mg/dl of calcium, and the pre-night 12-h sample contained 16.7 mg/dl of calcium. For the 12-h period during which the supplements were ingested the calcium level increased to 66.3 mg/dl and dropped to 50.1 mg/dl for the 12-h period following calcium supplementation.

The 4 pooled 12-h samples were then assayed to determine the effect on adherence. *E. coli* and epithelial cells were incubated in each of the 4 urine samples and then incubated in normal pooled urine (Table 4). At 24 and 12 h prior to ingestion of the calcium supplement, incubation in pooled urine of either *E. coli* (15.30 and 12.67 mean bacteria/cell) or epithelial cells (16.94 and 13.23 mean bacteria/cell) showed typical control values. For both *E. coli* and the epithelial cells there was a significant increase in the level of bacterial adherence for the 12-h period during (23.56 and 28.48 mean bacteria/cell) and following (28.02 and 23.66 mean bacteria/cell) calcium supplementation.

A time study was initiated to determine when the level of calcium peaked in the urine. After ingestion of 500 mg of calcium carbonate the level of calcium peaked in the urine 2 h, slowly declined at 4 h and continued to decline at 6 and 8 h. Based on this information, adherence studies were initiated in which *E. coli* and epithelial cells were incubated in normal urine, in urine 2 h after ingestion of 500 mg of calcium carbonate and 2 h after ingestion of 500 mg of calcium carbonate and 10 ounces of cranberry juice cocktail. In the normal urine there was a mean of 33.92 bacteria per cell. This value increased significantly ($P < 0.01$) to 46.12 bacteria per cell when the *E. coli* and epithelial cells were incubated in urine collected 2 h after ingestion of 500 mg of calcium carbonate. For the urine sample in which both calcium and cranberry juice cocktail were ingested, the mean bacteria per cell was 27.78 which is significantly lower ($P < 0.01$) than the sample supplemented with calcium alone and also significantly lower than the normal urine sample ($P < 0.05$).

Discussion

Since ions are known to interact with the surface of cells and occur with chelating agents that complex them in the urine, we undertook an examination of the effect of various ions on bacterial adherence. While sodium, potassium and chlorine ions had no effect on bacterial adherence and magnesium showed a minor decrease, calcium ions promoted a significant increase in bacterial adherence. This observation led to an in-depth examination of the role of calcium in promoting bacterial adherence since calcium is increasingly being suggested as a dietary supplement.

It was found from our in vitro experiments that 10 mM calcium chloride (400 mg/l of calcium) consistently increased bacterial adherence. This concentration is higher than that normally found in the urine, which ranges from about 50 to 250 mg/l for a 24 h sample [15]. In our sample we found that the concentration of calcium in normal urine during the 12-h daytime period was 337 mg/l and 167 mg/l for the 12-h nighttime period for an average of 252 mg/l. When 1,500 mg of calcium carbonate was taken during a 12-h daylight period, the calcium concentration increased to 660 mg/l and remained high at 500 mg/l for the next 12 h. This urine significantly increased bacterial adherence and is greater than the calcium concentration which consistently increased bacterial adherence in vitro. A two year study of 85 women with osteoporosis demonstrated an increase in the excretion of calcium occurred after dietary supplementation which calcium given as calcitriol [9]. Thus it is evident that supplementing the diet with 1,500 mg of calcium, a regimen that has been suggested by the National Institutes of Health to deter osteoporosis in postmenopausal women [16], can increase the concentration of calcium excreted in the urine to a level that significantly increases bacterial adherence.

The experiments in which the bacterial and epithelial cells were incubated in a calcium-containing medium and then shifted to a non or low calcium-containing medium suggested a carryover of calcium, probably on the surface of the cells. The wash sequence demonstrated that the calcium is associated more strongly with the surface of the epithelial cells since two incubations in 100 volumes of a non-calcium containing buffer were needed to eliminate the calcium-induced increase in adherence. These results suggest that a high concentration of calcium need not always be present in the urine for increased adherence to occur. When either type of cell is exposed to a high concentration of calcium ions enough of the calcium becomes associated with the surface of the cell to subsequently enhance bacterial adherence in the absence of the ion in the incubating medium. This is particularly true for the epithelial cells and suggests that even when high concentrations of calcium are not actively being excreted in the urine there may be enough calcium retained for a period of time on the epithelial cell surface to enhance bacterial adherence.

The traditional method for treating urinary tract infections is either via bacteriostatic or bacteriocidal agents [14]. In two recent publications we have demonstrated [11, 13], and Zafriri et al. [16] has confirmed, that

another approach that might be helpful would be the use of an anti-adherence agent like cranberry juice cocktail to prevent bacterial attachment. The juice is innocuous [10] and could be used over the long term to possibly treat or prevent recurrent urinary tract infections. In this study it was demonstrated that incubation of epithelial or bacterial cells in cranberry juice cocktail and 10 mM calcium completely eliminated the increase in adherence normally observed with 10 mM calcium. When ten ounces of the cocktail was taken with 500 mg of calcium carbonate the enhanced effect of the calcium was also completely reversed in urine collected two hours later. Presumably this was due to the presence in the urine of some factor or factors from the cranberry juice cocktail since we previously demonstrated that anti-adherence activity can be detected in the urine 2 h after drinking the cocktail [13].

In summary it is demonstrated that calcium, in concentrations higher than normally found in the urine, can significantly increase the adherence of uropathogenic *E. coli* to uroepithelial cells and this effect can be potentially reversed. This is of particular interest in view of current suggestion that dietary supplementation with calcium may be beneficial for the prevention and management of osteoporosis [1]. Based on the information presented here, it would appear that increased dietary uptake of calcium results in an increase in urinary excretion of calcium which can enhance the adherence of normal urinary pathogens to uroepithelial cells and thus increase the potential for urinary tract infection.

References

1. Culliton BJ (1987) Osteoporosis reexamined: complexity of bone biology is a challenge. Science 135:833
2. Duguid JP, Clegg S, Wilson MI (1979) The fimbrial and non-fimbrial haemagglutinins of *Escherichia coli.* J Med Microbiol 12:231
3. Fujita K, Yamamoto T, Yokota T, Kitagawa R (1989) In vivo adherence of type 1-fimbriated uropathogenic *Escherichia coli* to human ureteral mucosa. Infect Immun 57:2574
4. Hultgren SJ, Schwan AJ, Schaeffer AJ, Duncan JL (1986) Regulation of production of type 1 pili among urinary tract isolates O *Escherichia coli.* Infect Immun 54:613
5. Kallenius G, Svenson SB, Hultberg H, Mollby R, Helin I, Cedergren B, Winberg J (1982) Occurrence of P-fimbriated *Escherichia coli* in urinary tract infections. Lancet: 1369
6. Kisielius PV, Schwan WR, Amundsen SK, Duncan JL, Schaeffer AJ (1989) In vivo expression and variation of *Escherichia coli* Type 1 and P pili in the urine of adults with acute urinary tract infections. Infect Immun 57:1656
7. Kunin CM (1987) Detection, prevention and management of urinary tract infections, 4th edn. Lea and Febiger, Philadelphia, p 140
8. Leffler H, Svanborg-Eden C (1986) Glycolipids as receptors for *Escherichia coli* lectins or adhesins. In: Mirelman (ed) Microbial lectins and agglutinins: properties and biological activity. Wiley and Sons, New York, p 83
9. Ott SM, Chesnut CH (1988) Urine calcium response to calcitriol treatment in women with osteoporosis. Kidney Int 33:345
10. Prodromos PN, Brusch CA (1968) Cranberry juice in the treatment of urinary tract infections. Southwest Med 47:17
11. Schmidt DR, Sobota AE (1988) An examination of the anti-adherence activity of cranberry juice on urinary and nonurinary bacterial isolates. Microbios 55:173
12. Sharon N, Ofek I (1986) Mannose-specific bacterial surface lectins. In: Mirelman (ed) Microbial lectins and agglutinins: properties and biological activity. Wiley and Sons, New York, p 55
13. Sobota AE (1984) Inhibition of bacterial adherence by cranberry juice: potential use for the treatment of urinary tract infections. J Urol 131:1013
14. Stamm WF, Hooton TM, Johnson JR, Johnson C, Stapleton A, Roberts PL, Moseley SL, Fihn SD (1989) Urinary tract infections from pathogenesis to treatment. J Infect Dis 159:400
15. Wallach J (1986) Normal values: urine. In: Interpretation of diagnostic tests: a handbook synopsis of laboratory medicine. Little, Brown and Co, Boston, p 22
16. Zafriri D, Ofek I, Adar R, Pocino M, Sharon N (1989) Inhibitory activity of cranberry juice on adherence of type 1 and type P-fimbriated *Escherichia coli* to eucaryotic cells. Antimicrob Agents Chemother 33:92

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