ROLE OF CALCIUM SUPPLEMENTS IN THE PATHOGENESIS OF URINARY TRACT INFECTIONS

by

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ABSTRACT

ROLE OF CALCIUM SUPPLEMENTS IN THE PATHOGENESIS OF URINARY TRACT INFECTIONS

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It has been previously shown that calcium ions are able to reduce the anti-adherence activity of Tamm-Horsfall glycoprotein (THP). Upon duplication of this effect of calcium ions on THP, it was observed that calcium ions alone had a similar effect on the adherence of bacteria to exfoliated epithelial cells in humans. This study focuses on the role of excess calcium in relation to urinary tract infections. Also, the effect of calcium supplements on urinary excretion of calcium was investigated. A constant increase in bacterial adherence was observed as the concentration of calcium was increased whether the preincubation or incubation medium was phosphate buffered saline (PBS), sterile urine or a combination of both. An increase in the concentration of calcium to 10 mM or 20 mM more than doubled the number of bacteria adhering to exfoliated epithelial cells in vitro. Other monovalent and divalent ions that are abundant in urine were tested to see whether they affected bacterial adherence in the same manner as the calcium ions. Sodium and potassium ions did not promote bacterial attachment while magnesium ions

decreased the adherence of bacteria to exfoliated epithelial cells. The amount of calcium excreted in the urine after the ingestion of TUMS, an oral calcium supplement, is approximately equal to the concentration of 10 mM calcium that was previously shown to increase bacterial adherence *in vitro*. These results suggest that calcium supplements may increase the potential for urinary tract infections, particularly among postmenopausal women taking calcium supplements as prophylaxis against osteoporosis.

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CHAPTER I

INTRODUCTION

Although humans are constantly exposed to a wide variety of microorganisms in the environment, only a small proportion of these microbes are capable of interacting with the human host to induce infection. The human body provides a favorable environment for the growth of many microorganisms. However, since each part of the body differs in chemical and physical properties, certain microbes are more likely to inhabit specific body sites. The skin, oral cavity, respiratory tract, intestinal tract, and genitourinary tract provide a wide variety of microenvironments in which different microbes can grow selectively. But, only those microbes capable of colonizing these body surfaces by developing ways to overcome host defense mechanisms will survive and possible establish infection (Mims, 1987). When the body is invaded by pathogenic microbes, pathogens are confronted with a variety of nonspecific mechanisms to prevent infectious diseases. The nonspecific mechanisms which are usually effective against microbes in the respiratory and urinary tracts include the mucous membranes, coughing, sneezing, ciliary action, urination, peristalsis, and salivation (Cano and Colome, 1986). A mucociliary blanket covering most of the surface of the lower respiratory tract serves to protect the host from microbes. Microbes entering the

respiratory tract will get caught up in the mucus, be carried to back of the throat and then swallowed. Only those microbes capable of attaching to epithelial cells will be able to avoid the mucociliary mechanism. Bordetella pertussis, for example, appears to attach to respiratory epithelium and then is free to infect the normal lung causing whooping cough (Mims, 1977).

Normal flora, an antibacterial defense mechanism, protects the host against invading bacteria by consuming nutrients and preventing bacterial adherence to the host surface. For example, Escherichia coli, Fusobacterium, and Bacteroides make it difficult for Salmonella and Shigella to colonize the intestine and cause disease. In order to survive and possibly establish infection, these organisms must find a way to adhere to mucosal surfaces; if not, they will be devoid of nutrients and will be eliminated by the host's defense mechanisms (Svanborg-Edén et al., 1982).

Micturition, a primary anti-adherence defense mechanism, usually keeps the urethra relatively free of bacteria. As the urine flows through the urethra from the bladder, it washes out the urethral lumen and walls and carries with it many of the bacteria that have entered the urethra. Although most of the infected urine may be removed, a film of urine containing bacteria remains and continues to coat the bladder mucosa (Sobel, 1985). This film usually contains a sufficient amount of bacteria to induce colonization. A prerequisite for the colonization

of the urine is attachment of bacteria to the mucosal surface resulting in a urinary tract infection (Polito et al., 1987).

Urinary tract infections are the most common bacterial infections of people of all ages. The majority of these infections arise from the ascending movement of bacteria, that is by way of the urethra to the bladder and then up the ureters to the kidneys. A larger percentage of newborn males have urinary tract infections than do females because the lengthened urethra may be vulnerable during the birthing process (Cano and Colome, 1986). In adult females, the urethra is so short (approximately 5 cm) that the flushing mechanism of the bladder is not as effective in removing bacteria as it is in males (Mims, 1987). The prevalence of urinary tract infections has been shown to be 14 times more frequent in adult females than males (Mims, 1987). In contrast, a higher incidence of urinary tract infection is observed for males over the age of 65. Men over 65 are more susceptible to urinary tract infections possibly due to prostatic disease and urethral catheterization (Mims, 1987). Since urinary tract infection is still a common health problem of today, numerous studies have attempted to isolate the major virulence factor or factors involved in initiating this discomforting disease. It appears that attachment of bacteria to the mucosal surface is a prerequisite for

infection (Svanborg Edén et al., 1976; Schaeffer et al., 1987).

Several mechanisms appear to be important in bacterial adherence to various body tissues and surfaces. The capsules of many gram-negative bacteria have been shown to confer resistance to phagocytosis. Richardson and Sadoff (1977) observed that encapsulated gonococci were more resistant to phagocytosis than nonencapsulated organisms. The capsule appears to hinder the attachment of phagocytes to the bacterial surface by electrostatic repulsion. Therefore, capsules may be major virulence factors by nature of their antiphagocytic activity. In the rat model of intra-abdominal sepsis, only encapsulated Bacteroides produced abscesses. It was then concluded that the virulence of Bacteroides appears to be related to the presence of a capsule (Onderdonk et al., 1977).

The glycocalyx, a filamentous network of polysaccharide that extends from the surface of many bacteria and cells, allows bacteria a means of binding and channeling nutrients into their cells. The function of the glycocalyx is similar to the bacterial capsule in that it offers bacteria protection from phagocytosis and may serve as a virulence factor. Many diseases are caused by bacteria that are able to synthesize a glycocalyx. Dental caries result from the action of bacteria on the teeth, the most common of which is Streptococcus mutans. This bacteria contains a high molecular weight polysaccharide,

glucan, which serves as the mode of attachment. The initial step in the development of caries is the deposition of plaque on the teeth which serves as a reservoir for bacteria. Therefore, the ability of S. mutans to attach appears to be related to its ability to produce a glycocalyx (Brock et al., 1984). Smith and Huggins (1978) demonstrated that glycocalyx antigens among strains of E. coli increased their ability to colonize the small intestine and cause diarrhea in pigs and calves. Several species of bacteria isolated from urine have been shown to contain glycocalyces. To date no direct correlation has been found relating the incidence of urinary tract infection and the adherence of glycocalyx-coated E. coli to exfoliated epithelial cells.

Bacterial adherence, mediated through specific adhesive organelles, fimbriae or pili, has been implicated as a virulence factor in many types of infections. Duguid and Gillies (1957) observed that piliated Shigella flexneri adhered to human intestinal epithelial cells in vitro. Bacteria with few or no pili were non-adherent. With this observation, they suggested that the virulence of piliated S. flexneri was associated with the ability to attach to epithelial cells. The suggestion that bacterial adherence represents a virulence factor was further supported by Duguid et al., (1976). They reported that piliated strains of Salmonella typhimurium caused more infections and deaths than nonpiliated strains in mice. Svanborg-Edén and

Hansson (1978) found a significant correlation between the presence of fimbriae on E. coli and the ability of the bacteria to adhere to human uroepithelial cells. Among the 12 strains tested, none with adhesive ability lacked fimbriae while only a few of the nonadhering strains contained fimbriae. When the bacteria were heated or subjected to chemical treatment to remove fimbriae, a loss in adhesion paralleled the loss of fimbriae. These findings suggest that fimbriae or substances coappearing with fimbriae may act as mediators of adherence for bacteria to human exfoliated epithelial cells in urinary tract infections.

Although urinary tract infections are caused by many species of microorganisms, E. coli is the most commonly isolated pathogen. In E. coli strains isolated from patients with urinary tract infections, there are specific molecular structures called fimbriae on the surface of these bacteria that recognize and bind to specific glycoconjugates on the uroepithelial cell surface (Ofek et al., 1977). The presence of specific receptor sites on exfoliated epithelial cells is essential for microbial infectivity. If the host does not express the correct receptor, binding and subsequent colonization and infection cannot take place.

Several types of fimbriae are formed by pathogenic enterobacteria and confer upon the bacterial cells the ability to adhere to cells. Even though most fimbriae

share many structural and biochemical similarities, they differ with respect to their antigenic and functional properties. A wide variety of adhesins have been identified and classified on the basis of antigenic type and receptor specificity. The first adhesin identified on uropathogenic E. coli was a mannose-sensitive fimbriae. Duguid et al. (1980) and Ofek et al. (1977) reported that purified type 1 fimbriae (mannose-sensitive) of E. coli agglutinate mannose-containing yeast cells and guinea pig erythrocytes and bind to several other mammalian cell types. They found that the binding of E. coli to each of these cell types was specifically blocked by solutions of D-mannose and its derivatives. This inhibition implies that the receptor site for the bacteria appears to be antigenically similar to the simple sugar mannose. Although type 1 fimbriae can attach to urinary mucus, a specific role for this adhesin in E. coli uropathogenicity has not been clearly defined (Orskov and Orskov et al., 1980). Fader et al. (1979) demonstrated that a uropathogenic strain of Klebsiella pneumoniae adhered to rat bladder epithelial cells in vitro via mannose-sensitive fimbriae. Therefore, it appears that mannose-sensitive adhesins may contribute to E. coli infectivity in the urinary tract.

The major type of adhesin identified on uropathogenic E. coli in humans is the P fimbriae (Pere et al., 1987; Parkkinen et al., 1988). These P fimbriae bind

specifically to α-D-Galactosyl-(1-4)-β-D-Galactopyranoside residues of globohexosylceramides present on exfoliated epithelial cells. The molecular specificity of binding was demonstrated by the complete inhibition of the fimbrial binding by globotriose, a receptor analog for P fimbriae. In contrast, methyl-α-D-mannoside, a receptor analog for type 1 fimbriae, had no effect on the P fimbriae (Korhonen et al., 1986). Therefore, P fimbriae are classified as mannose-resistant fimbriae. Several studies have reported a direct correlation between virulence of uropathogenic E. coli in humans and the presence of P fimbriae (Parkkinen, et al., 1988; Pere et al., 1987). P fimbriated E. coli has been isolated from young girls experiencing acute urinary tract infection (Pere et al., 1987).

An adhesin that rarely occurs in uropathogenic E. coli strains is called S fimbriae. These fimbriae are inhibited by oligosaccharides containing sialyl galactoside sequences such as NeuAc(α2-3)Gal(β1-4)Glc (Parkkinen et al., 1988). This inhibition implies that sialyloligosaccharides are part of the host receptor. Although distinct in their receptor structure, S fimbriae bind to the same epithelial tissues in the human urinary tract as P fimbriae, but do not lead to infection. This suggests that bacterial adherence to specific receptor sites on the exfoliated epithelial cells does not necessarily lead to infection, some other factors may be involved (Parkkinen et al. 1988).

Many bacteria are capable of expressing more than one type of adhesin. In fact they may synthesize several different kinds of fimbriae at the same or different times. Svanborg-Edén et al. (1978) observed that patients with recurrent urinary tract infections had more bacteria in the urethral and vaginal regions than women without a history of urinary tract infections. Similarly, Schaeffer et al. (1981) observed an increase in adherence of E. coli to exfoliated vaginal epithelial cells in women prone to infection as compared to healthy control subjects. Therefore, colonization seems to be an important virulence factor in recurrent urinary tract infection. Källenius et al. (1980) demonstrated that uroepithelial cells among persons suffering from recurrent urinary tract infections bind more frequently to P-fimbriated E. coli than do uroepithelial cells from healthy individuals. Of 97 children with urinary tract infection, 91 percent with pyelonephritis expressed the receptors for P fimbriae, while only 19 percent of the children with cystitis and 14 percent with asymptomatic bacteriuria expressed the receptors for P fimbriae. Such data suggests that individuals susceptible to recurrent urinary tract infections may have an increased receptivity of their uroepithelial cells for adhering bacteria due to an increased density of receptor sites (Korhonen et al., 1986).

There is a strong relationship between adherence and the seriousness of the infection, whether the adherence is mediated by specific receptors (type 1 pili and P fimbriae) or by other specific bacterial antigens (glycocalyx).

Kaye (1968) asserts that normal human urine contains antibacterial defense mechanisms that exert a protective effect against urinary tract infections. The most important factors appear to be a high urea concentration, extremes of osmolality, high organic acid concentration, and a low pH level.

Another important defense mechanism in urine is Tamm-Horsfall glycoprotein (THP) or uromucoid. This glycoprotein is secreted by the tubular cells of the kidney's ascending loop of Henle and is rich in mannose oligosaccharide residues. These mannose-rich residues originally were thought to serve as attachment sites for type 1-fimbriated E. coli (Sobel, 1985). In this way the bacteria would be prevented from binding to the exfoliated epithelial cells and cleared from the bladder during micturition. However, a recent study by Parkkinen et al. (1988) has shown that THP is a major inhibitor of S fimbriae rather than type 1-fimbriated E. coli. In fact, they found that type 1-fimbriated bacteria were inhibited by low-molecular-weight compounds present in normal human urine.

Calcium-containing stones can obstruct the urinary tract resulting in urinary tract infections. Calcium, a

major constituent of 90% of all renal calculi, is most commonly found in the form of calcium oxalate or calcium phosphate (Epstein, 1968). Patients who form calcium stones tend to have higher urinary excretions of calcium than non-stone formers (Robertson et al., 1968; Bulusu et al., 1970).

Calcium is important in many metabolic functions. The body relies on calcium for the growth and maintenance of bones and teeth, in addition, calcium aids in blood clotting and muscle contraction. The average Recommended Daily Allowance (RDA) of calcium for adults is 800 mg/day on a 1400 calorie diet (Nutrition in Health and Disease, 1982). On a low calcium diet 10% to 40% of dietary calcium is excreted in the urine (Tietz, 1970). Bradley and Benson (1974) determined the normal concentration of calcium in the urine of healthy individuals as 0.5 mM to 6.0 mM. A defect in the calcium metabolism may lead to an increase in urinary calcium excretion. Osteolytic bone diseases, renal tubular acidosis, and hypercalciuria are a few of the common disorders that result in an increase in calcium excretion.

The initial aim of this study was to investigate the role of urinary excretion of excess calcium in relation to urinary tract infections. Since postmenopausal women are particularly susceptible to osteoporosis, physicians currently recommend that they consume calcium supplements in excess of the daily RDA of calcium. This study examines

the effect of excess calcium in urine due to ingestion of calcium supplements and its potential role in urinary tract infections.

CHAPTER II

MATERIALS 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 organisms were previously identified as *Escherichia coli* by API 20E strips (Petruna, 1987) and shown to contain P fimbriae. For long-term storage the bacteria were transferred to agar talls of brain heart infusion (BHI), grown at 37° C for 48 hours, and then stored at 2-6° C. When bacteria were needed for testing, they were transferred to tryptic soy broth and grown for 48 hours at 37° C.

Exfoliated Epithelial Cells

Exfoliated epithelial cells were obtained from the sediment of freshly voided urine. Urine specimens were obtained from a healthy female with no known history of urinary tract infection. A 30 mL sample of urine was centrifuged at 1000xg for 10 minutes to harvest epithelial cells. The supernatant was discarded and the pellet was resuspended in 0.01 M phosphate buffered saline (PBS) at a pH of 7.2, unless otherwise stated.

Bacterial Adherence Assay

The method used in these experiments was that of Sobota (1984) and is a modification of the technique

described by Parsons, et al. (1980). 20 mL of E. coli from a 48 hour culture and 30 mL of freshly voided urine were centrifuged at 1000xg for 10 minutes. The supernatants were discarded and the E. coli pellet was washed one time in 10 mL of PBS, pH 7.2. Both pellets were resuspended in PBS unless otherwise stated. Then, 1 mL samples of E. coli were incubated with 1 mL of different concentrations of calcium chloride ($CaCl_2$) for 10 minutes at 37° C in a water bath. The exfoliated epithelial cells were maintained at 37° C to preserve the structure of the cells. Following preincubation, the treated E. coli was centrifuged, the supernatant was discarded, and 1 mL of exfoliated epithelial cells in PBS was added. These mixtures were incubated for 30 minutes at 37° C along with a 1 mL sample of epithelial cells in PBS without added bacteria (baseline control) and a 1 mL sample of untreated E. coli with epithelial cells (control). Following incubation, 1 mL of the cell-bacteria mixture was removed using a tuberculin syringe. The tuberculin syringe containing the 1 mL sample was then attached to a filter apparatus containing a 8 um polycarbonate membrane filter (Nucleopore). The samples were filtered and washed three times with 10 mL of deionized water to remove any non-adherent bacteria. The filters were removed and placed face down onto glass slides and allowed to dry. After the filters had dried completely, they were then removed and the adhering epithelial cells and E. coli were gram-stained. A total of

50 exfoliated epithelial cells was scored for adherent bacteria using the oil-immersion objective of a bright-field microscope. Adherence was recorded as the average number of bacteria per epithelial cell. If the average number of bacteria per epithelial cell in the baseline control exceeded five bacteria per cell, the results were omitted (Sobota, 1984).

Scanning electron microscopy

Following completion of the standard bacterial adherence assay in which E. coli was preincubated with 20 mM calcium and then mixed with exfoliated epithelial cells and incubated in sterile urine, the 1 mL sample was prepared for electron microscopy. After 1 hour of fixation in 5 mL of 2% gluteraldehyde, the mixture was washed twice with 10 mL PBS and dehydrated through the alcohol series, 5 min. in each. The filter paper was removed and critical point dried. After drying, the filter paper was cut into pieces, mounted on silver painted stubs, and gold sputter coated.

Analysis of calcium, magnesium, sodium, and potassium ions on bacterial adherence

The standard bacterial adherence assay was performed, but instead of incubating with different concentrations of $CaCl_2$, 1 mL of 10 mM concentration of calcium chloride, magnesium chloride crystals, sodium chloride, and potassium chloride crystals was used.

Analysis of urine after dietary calcium supplementation

The method used in this experiment was that of Hunt and Johnson (1983). A healthy female with no history of urinary tract infection was placed on a non-dairy diet of the same foods for three consecutive days. On the second day, first morning urine was discarded and then urine was collected continuously until bedtime. This urine was labelled pre-day no calcium. The following morning, the first morning urine was collected and labelled pre-night no calcium. Then, a total of three "TUMS" each consisting of 500 mg calcium carbonate was taken either with or following meals. Urine was collected over a 24 hour period and marked as post-day with calcium. The following morning, the first morning specimen was collected and labelled post-night with calcium. The urinary output of calcium was measured by a Sigma diagnostic kit (No. 587). The samples were quantified spectrophotometrically at 575 nm using a Bausch and Lomb Spectronic 20. Also, a bacterial adherence assay was performed in which 1 mL of the urine samples was used in place of the different concentrations of calcium chloride.

Time study analysis of urine after dietary calcium supplementation

The method used in this experiment is a modification of the technique described by Hunt and Johnson (1983). A healthy female with no prior history of urinary tract infection was placed on a non-dairy diet of the same foods for three consecutive days. On the second day, first

morning urine was discarded and then urine was collected approximately every 2 hours until bedtime. The next morning, first morning urine was collected. Then a total of three "TUMS" was taken either with or following a meal. Urine was collected every 2 hours until bedtime and then again the following morning. The urinary output of calcium was measured by a Sigma diagnostic kit (No. 587) and quantified spectrophotometrically at 575 nm.

Statistical Analysis

The mean adherence values were compared to the controls and to each other by using the two-tailed Student "t" test.

CHAPTER III

RESULTS

Adherence of E. coli to exfoliated epithelial cells

To demonstrate the attachment of E. coli to the surface of exfoliated epithelial cells a sample of epithelial cells with adherent bacteria was visualized via scanning electron microscopy. E. coli was incubated in sterile urine supplemented with 20 mM calcium. Exfoliated epithelial cells were then added and incubation was continued to permit attachment. The preparation was then washed and prepared for electron microscopy. An electron micrograph of a typical cell with adherent E. coli can be observed in Figure 1.

Effect of calcium on the adherence of E. coli to exfoliated epithelial cells

It can be observed from Table 1, that if a mix of E. coli and exfoliated epithelial cells is incubated in PBS the mean number of bacteria that adhere to each bacterial cell is approximately 24. This differs significantly from the baseline control in which no E. coli was included during the incubation and the mean number of adhering bacteria was 0.06 per cell. If increased levels of calcium are included in the incubation medium there is an additional increase in the level of adhering bacteria. In Test A of Table 1, increasing the level of calcium to 20 mM more than doubles the number of adhering bacteria. The

number of adhering bacteria also increased when 5 mM calcium was added to the incubation medium (Test B, Table 1). In both cases there was a significant increase in the level of bacterial adherence over control levels.

Since normal urine is known to contain ionized calcium and also substances that will complex calcium ions (Elliot and Eusebio, 1965; Vogel et al., 1984), sterile urine was substituted for the PBS as the incubation medium. The results appear in Table 2. Again when E. coli was included in the incubation medium (control) there was a significant increase in the number of adhering bacteria as compared to the baseline control in which no E. coli was included in the incubation medium. It can also be observed that the baseline control and control levels of bacterial adherence were slightly lower than that observed in the previous experiment. When the level of calcium in the incubation medium was increased to 20 mM, again there was an approximate two-fold increase in the number of adhering bacteria. This was significantly different from control values. An increase in the level of calcium to 5 mM also resulted in a significant increase in bacterial adherence.

Adherence of E. coli to exfoliated epithelial cells after preincubation in a medium containing various concentrations of calcium

In order to determine if calcium had to be continually present in the medium to increase bacterial adherence, E. coli or exfoliated epithelial cells were preincubated in a

calcium-containing medium and then shifted to another medium to allow adherence. The results appear in Tables 3, 4, 5, and 6. In Tables 3 and 4 either E. coli or exfoliated epithelial cells were preincubated in PBS plus calcium followed by incubation for adherence in sterile urine. As in the previous results, control values were significantly different from baseline controls which contained no E. coli in the incubation medium. When E. coli was preincubated with either 20 or 10 mM calcium and then mixed with the exfoliated epithelial cells and incubated in sterile urine, there was a significant increase in bacterial adherence (Table 3, Tests A and B). If the level of calcium in the preincubation medium was decreased to 5 mM and 0.1 mM no increase in bacterial adherence was observed (Table 3, Tests C and D). A similar pattern was observed when exfoliated epithelial cells were preincubated in various concentrations of calcium and then transferred to sterile urine and mixed with E. coli. At concentrations of 10 mM and 20 mM an increase in bacterial adherence was observed (Table 4, Tests A and B), whereas at concentrations of 5 and 0.1 mM no increase in bacterial adherence was observed (Table 4, Tests C and D).

If after preincubation in PBS and calcium the E. coli and exfoliated epithelial cells were allowed to interact in PBS rather than sterile urine, the results were similar. Preincubation of E. coli in PBS followed by incubation with exfoliated epithelial cells in 20 mM and 5mM calcium

resulted in a significant increase in bacterial adherence (Table 5, Tests A and B). Preincubation in 1 or 0.1 mM calcium showed no significant increase in bacterial adherence (Table 5, Tests C and D). An increase in bacterial adherence was also observed when exfoliated epithelial cells were preincubated in 20 mM calcium (Table 6, Test A) but no increase in adherence was observed when the preincubation medium contained 5, 1 or 0.1 mM calcium (Table 6, Tests B, C, and D). In our experience, a concentration of about 10 mM calcium or greater was necessary to consistantly increase bacterial adherence *in vitro*. In one instance reported here, (Table 5, Test B) a concentration of 5 mM calcium resulted in an increase in bacterial adherence.

The results from Tables 3, 4, 5, and 6 are summarized in Figure 2. It can be observed that in all cases, irrespective of the preincubation medium or the incubation medium for adherence, a constant increase in adherence was observed as the concentration of calcium was increased. It is also evident that preincubation of *E. coli* in a calcium-containing medium consistently resulted in a higher level of bacterial adherence as compared to corresponding experiments with exfoliated epithelial cells.

In order to observe the effect of calcium on bacterial adherence as it might occur *in vivo*, sterile urine was used as the preincubation and incubation media. The results appear in Table 7. The mean number of bacteria that adhere

to each exfoliated epithelial cell, in the control, was approximately 26. This is significantly different from the baseline control to which no bacteria were added. Preincubation of E. coli in sterile urine plus 10 mM calcium followed by incubation in sterile urine resulted in a significant increase in bacterial adherence to exfoliated epithelial cells as compared to the control. When the level of calcium was decreased to 5 mM, no increase in bacterial adherence was observed.

A comparative evaluation of calcium, magnesium, sodium, and potassium ions on adherence

To determine if an excess of other urinary ions has an effect on the adherence of bacteria to exfoliated epithelial cells, E. coli or exfoliated epithelial cells were preincubated in PBS containing the various ions followed by incubation in sterile urine. The results appear in Tables 8 and 9. The mean adherence for the baseline control was 0.04 bacteria per exfoliated epithelial cell while the mean adherence for the control was significantly different with a mean adherence of 16.68. When E. coli was preincubated with a concentration of 10 mM calcium and then mixed with the exfoliated epithelial cells and incubated in sterile urine, there was a significant increase in bacterial adherence (Table 8, Test A). Preincubation of E. coli with 10 mM sodium and potassium showed no significant increase in adherence (Table 8, Tests C and D). On the other hand, preincubation of E. coli with

a concentration of 10 mM magnesium significantly reduced the amount of bacteria adhering to epithelial cells (Table 8, Test B). A similar pattern was observed when exfoliated epithelial cells were preincubated in a 10 mM concentration of the different ions and then transferred to sterile urine and mixed with E. coli. An increase in bacterial adherence was observed when exfoliated epithelial cells were preincubated with calcium (Table 9, Test A), but no increase in adherence was observed for sodium and potassium (Table 9, Tests C and D). Preincubation of exfoliated epithelial cells with 10 mM magnesium significantly reduced the number of bacteria adhering to exfoliated epithelial cells (Table 9, Test B).

Evaluation of urine after dietary calcium supplementation

It can be observed from Table 10 that the concentration of calcium in 24 hour urine samples collected and measured by Sigma diagnostic kit is greater after the ingestion of TUMS (66 mg/dL and 50 mg/dL) than before calcium supplements were taken (33.7 mg/dL and 16.7 mg/dL). The concentration of calcium equivalent to 10 mM calcium chloride, which significantly increased bacterial adherence, was calculated to be 40.08 mg/dL.

Based on these results, a bacterial assay was performed using these 24 hour urine samples. The results appear in Tables 11 and 12. When E. coli was preincubated in PBS plus the urine collected before and after consumption of TUMS followed by incubation in sterile

urine, a significant increase in bacterial adherence was observed for the urine labelled post-day and post-night with calcium as compared to the control (Table 11, Tests C and D). In contrast, the effect on bacterial adherence with the urine labelled pre-day and pre-night no calcium was similar to the control (Table 11, Tests A and B). Similar results were observed when exfoliated epithelial cells were preincubated in PBS plus urine collected before and after TUMS consumption and then incubated in sterile urine. In Table 12, the mean number of bacteria that adhered to each bacterial cell is 17.38 which is significantly different from the baseline control. Again, no change in bacterial adherence was noted for the urine labelled pre-day and pre-night no calcium in relation to the control. A significant increase in the binding of E. coli to exfoliated epithelial cells was observed when exfoliated epithelial cells were preincubated with urine labelled post-day and post-night with calcium.

In order to determine the specific time in which the calcium level is highest in the urine, before or after consumption of TUMS, urine was collected approximately every two hours for two days. The results are presented in Table 13. The concentration of calcium before the consumption of TUMS (control) remains relatively stable, then peaks around 4:30 pm, and finally peaks again at 10:30 pm. On the other hand, each time that a tablet was ingested a sharp increase in the level of calcium was

observed as compared to the control, except after 6:30 pm.

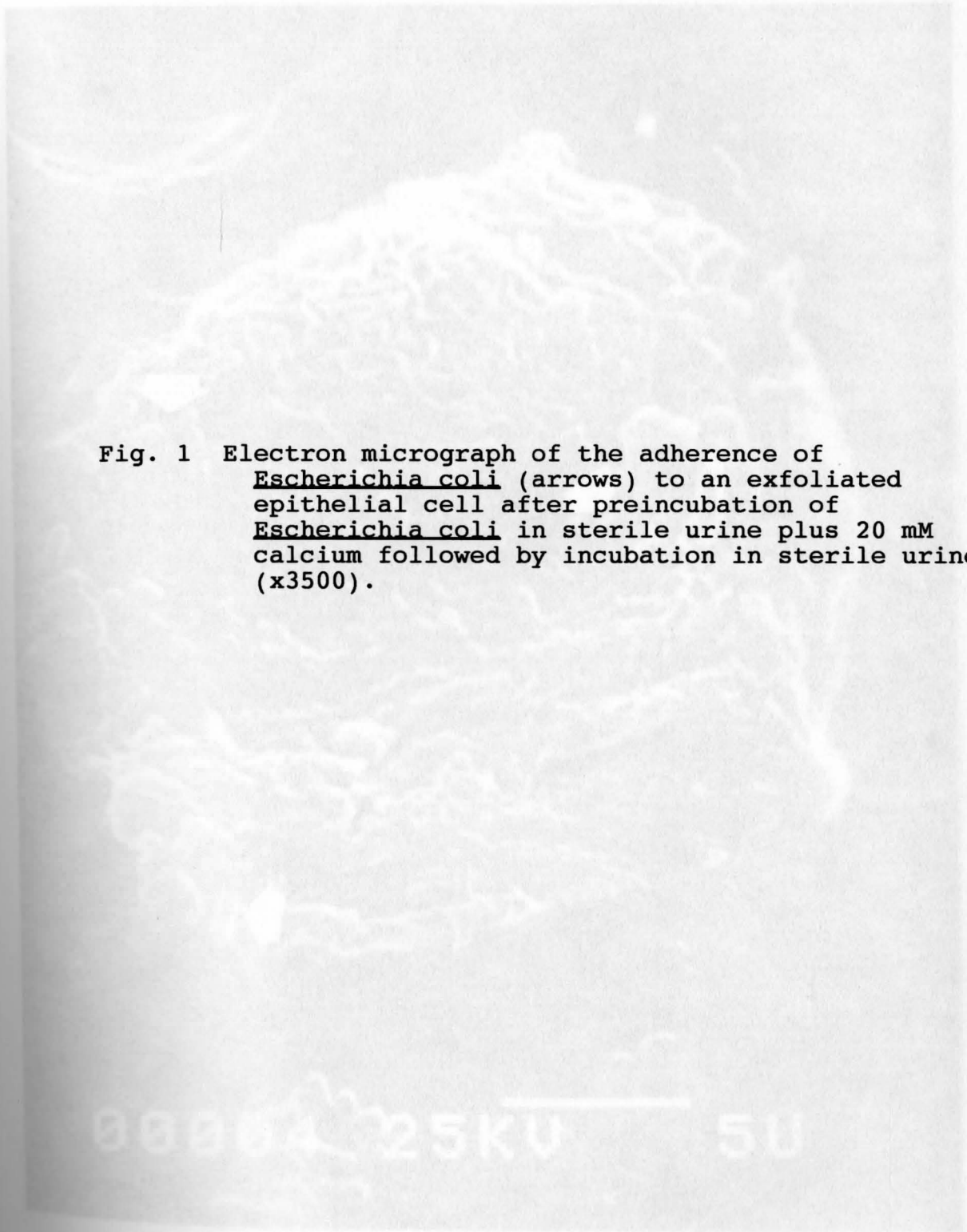

Fig. 1 Electron micrograph of the adherence of Escherichia coli (arrows) to an exfoliated epithelial cell after preincubation of Escherichia coli in sterile urine plus 20 mM calcium followed by incubation in sterile urine (x3500).

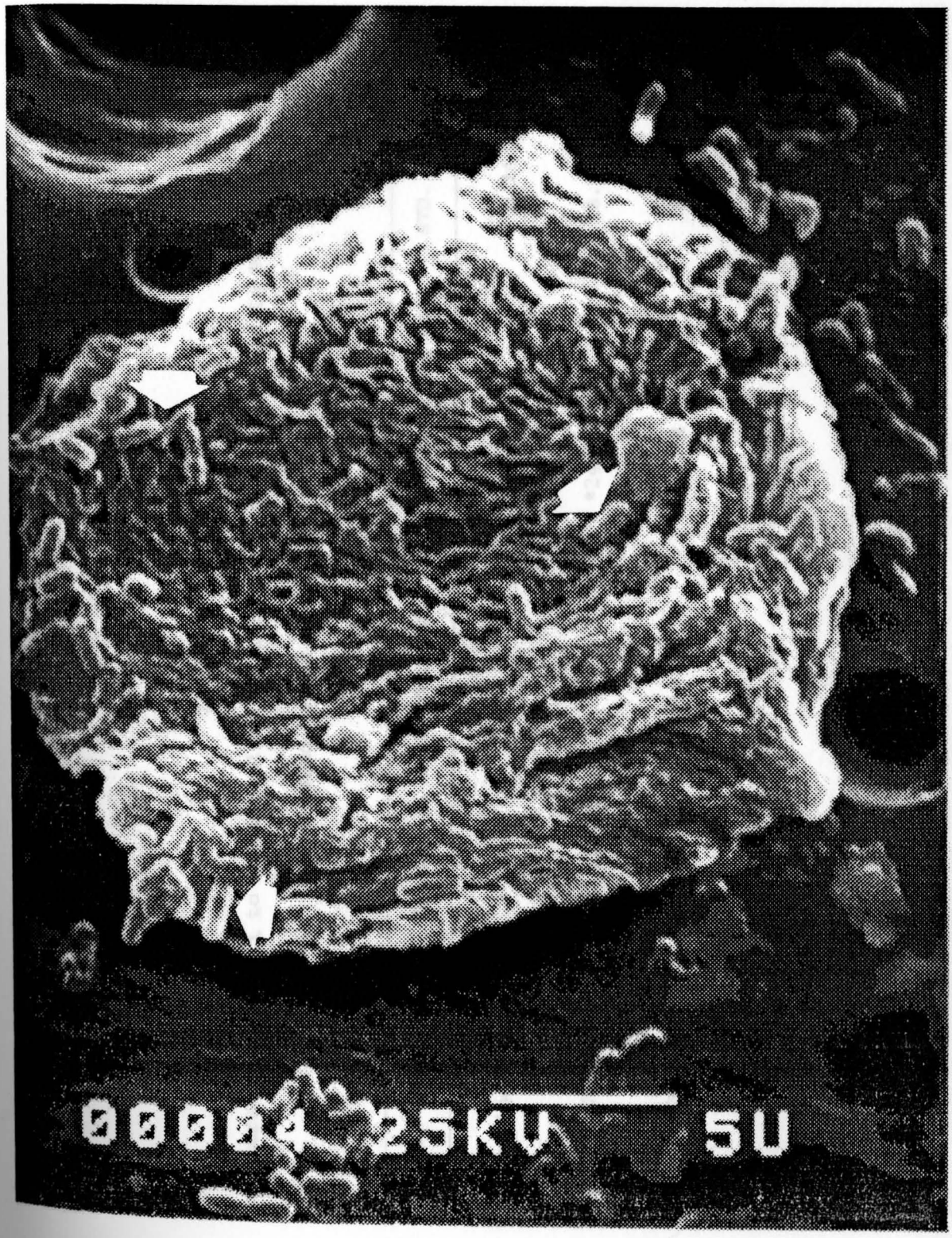
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TABLE 1

Adherence of *E. coli* to exfoliated epithelial cells after incubation in PBS with increased levels of calcium.

Group	Epithelial cell treatment	Mean Bacteria per Cell	STD. DEV.
Baseline Control	None	0.06	0.24
Control	*E. coli*	*24.26	18.83
Test A	*E. coli* + 20mM Ca++	**48.14	6.21
B	*E. coli* + 5mM Ca++	**32.33	17.34

*Significantly different from baseline control ($p<0.01$).

**Significantly different from control ($p<0.05$).

TABLE 2

Adherence of *E. coli* to exfoliated epithelial cells after incubation in urine with increased levels of calcium.

Group	Epithelial cell treatment	Mean Bacteria per Cell	STD. DEV.
Baseline Control	None	0	0
Control	*E. coli*	*19.20	16.24
Test A	*E. coli* + 20mM Ca++	**42.14	11.87
B	*E. coli* + 5mM Ca++	**39.50	13.21

*Significantly different from baseline control ($p<0.01$).

**Significantly different from control ($p<0.05$).

TABLE 3

Effect of preincubation of *E. coli* in PBS containing various concentrations of calcium followed by incubation with exfoliated epithelial cells in sterile urine.

Group	Epithelial Cell Treatment	Mean Bacteria per Cell	STD. DEV.
Baseline Control	None	0	0
Control	*E. coli*	*7.79	14.72
Test A	*E. coli* + 20mM Ca++	**35.78	17.23
B	*E. coli* + 10mM Ca++	**27.66	18.29
C	*E. coli* + 5mM Ca++	2.96	5.39
D	*E. coli* + 0.1mM Ca++	2.62	4.38

*Significantly different from baseline control ($p<0.01$).

**Significantly different from control ($p<0.05$).

TABLE 4

Effect of preincubation of exfoliated epithelial cells in PBS containing various concentrations of calcium followed by incubation with *E. coli* in urine.

Group	*E. coli* Treatment	Mean Bacteria per Cell	STD. DEV.
Baseline Control	None	0	0
Control	*E. coli*	*7.79	14.72
Test A	Epi. cells + 20mM Ca++	**20.96	17.65
B	Epi. cells + 10mM Ca++	**17.66	19.17
C	Epi. cells + 5mM Ca++	3.76	9.60
D	Epi. cells + 0.1mM Ca++	1.12	2.40

*Significantly different from baseline control ($p<0.01$).

**Significantly different from control ($p<0.05$).

TABLE 5

Effect of preincubation of *E. coli* in PBS containing various concentrations of calcium followed by incubation with exfoliated epithelial cells in PBS.

Group	Epithelial Cell Treatment	Mean Bacteria per Cell	STD. DEV.
Baseline Control	None	0	0
Control	*E. coli*	*4.56	11.45
Test A	*E. coli* + 20mM Ca++	**40.80	15.57
B	*E. coli* + 5mM Ca++	**9.12	11.78
C	*E. coli* + 1mM Ca++	1.10	1.96
D	*E. coli* + 0.1mM Ca++	1.06	2.74

*Significantly different from baseline control ($p<0.01$).

**Significantly different from control ($p<0.05$).

TABLE 6

Effect of preincubation of exfoliated epithelial cells in PBS containing various concentrations of calcium followed by incubation with *E. coli* in PBS.

Group	*E. coli* Treatment	Mean Bacteria per Cell	STD. DEV.
Baseline Control	None	0	0
Control	*E. coli*	*4.56	11.45
Test A	Epi. cells + 20mM Ca++	**21.86	20.09
B	Epi. cells + 5mM Ca++	7.84	12.24
C	Epi. cells + 1mM Ca++	2.10	4.39
D	Epi. cells + 0.1mM Ca++	0.77	1.67

*Significantly different from baseline control ($p<0.01$).

**Significantly different from control ($p<0.05$).

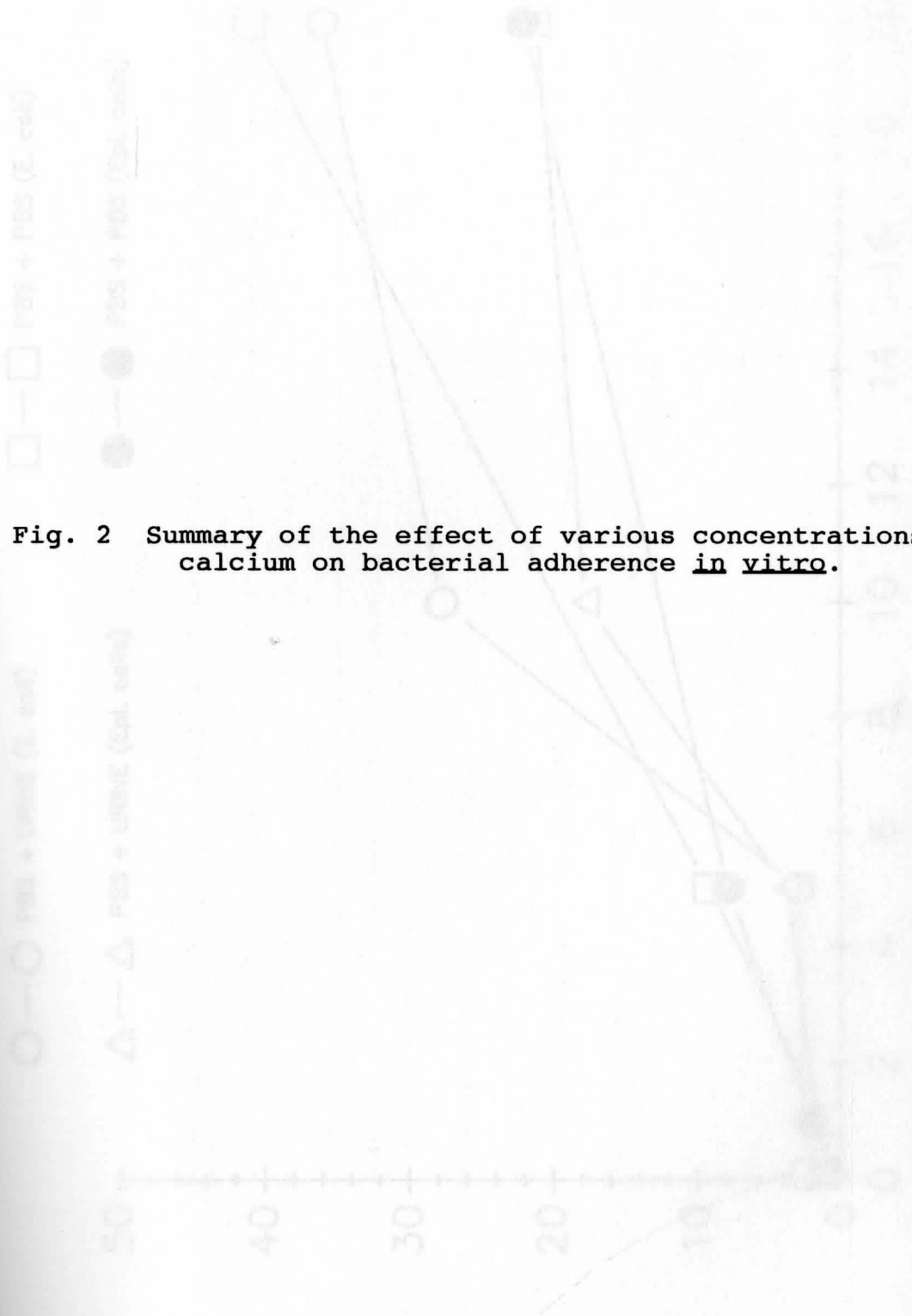


Fig. 2 Summary of the effect of various concentrations of calcium on bacterial adherence *in vitro*.

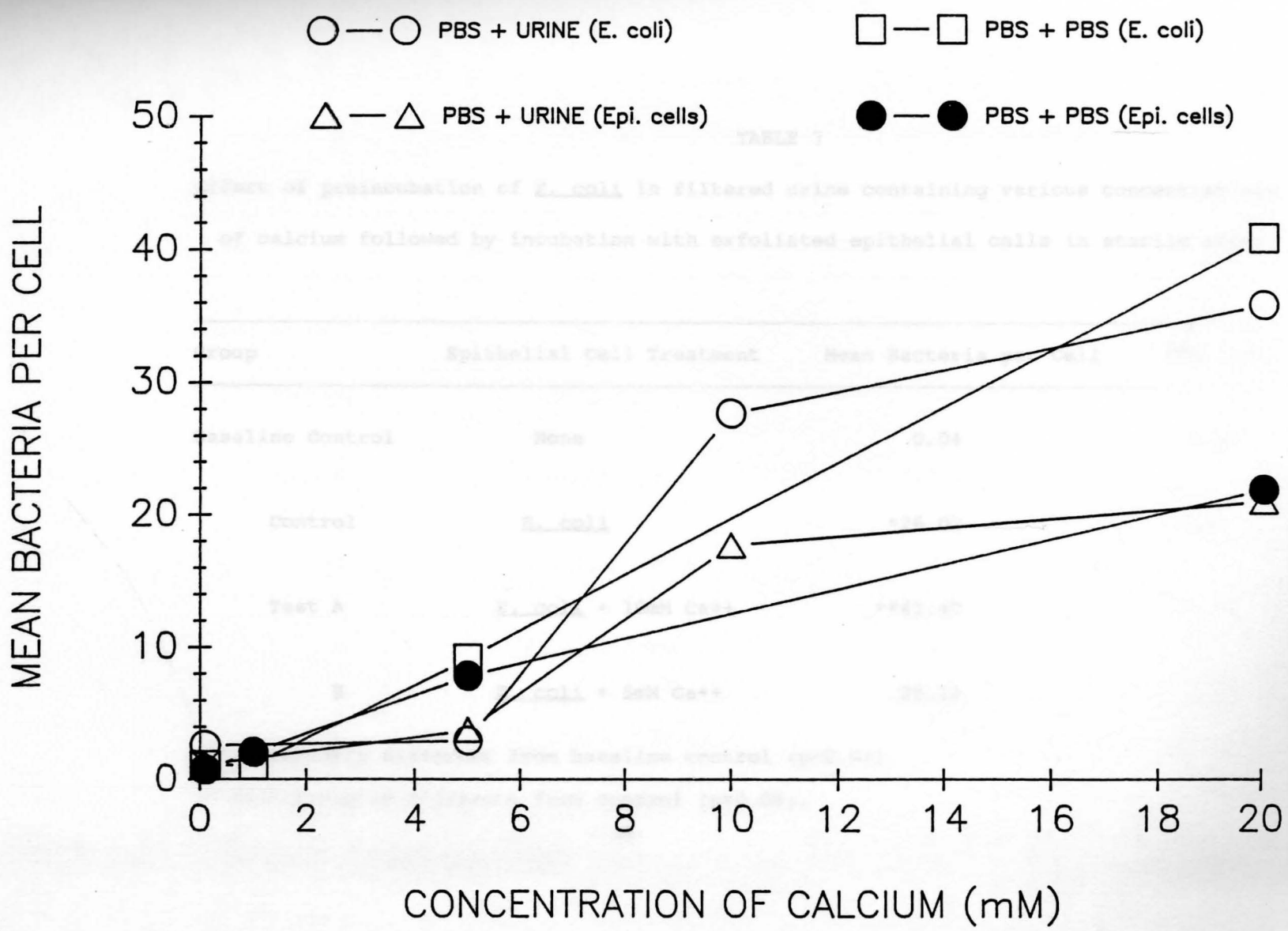

○—○ PBS + URINE (E. coli)
□—□ PBS + PBS (E. coli)
△—△ PBS + URINE (Epi. cells)
●—● PBS + PBS (Epi. cells)
MEAN BACTERIA PER CELL
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
CONCENTRATION OF CALCIUM (mM)

TABLE 7

Effect of preincubation of *E. coli* in filtered urine containing various concentrations of calcium followed by incubation with exfoliated epithelial cells in sterile urine.

Group	Epithelial Cell Treatment	Mean Bacteria per Cell	STD. DEV.
Baseline Control	None	0.04	0.28
Control	*E. coli*	*26.02	16.41
Test A	*E. coli* + 10mM Ca++	**41.40	12.49
B	*E. coli* + 5mM Ca++	25.12	15.90

*Significantly different from baseline control ($p<0.01$).

** Significantly different from control ($p<0.05$).

TABLE 8

A comparative evaluation of calcium, magnesium, sodium, and potassium ions on adherence.

Group	Epithelial Cell Treatment	Mean Bacteria per Cell	STD.DEV.
Baseline Control	None	0.04	0.20
Control	*E. coli*	*16.68	14.97
Test A	*E. coli* + Ca++	**35.14	17.63
B	*E. coli* + Mg++	**8.39	12.82
C	*E. coli* + Na+	18.95	17.68
D	*E. coli* + K+	10.91	15.91

*Significantly different from baseline control ($p<0.01$).

**Significantly different from control ($p<0.05$).

TABLE 9

A comparative evaluation of calcium, magnesium, sodium, and potassium ions on adherence.

Group	*E. coli* Treatment	Mean Bacteria per Cell	STD. DEV.
Baseline Control	None	0.04	0.20
Control	*E. coli*	*16.68	14.97
Test A	Epi. cells + Ca++	**42.80	11.12
B	Epi. cells + Mg++	**10.11	12.88
C	Epi. cells + Na+	11.18	12.12
D	Epi. cells + K+	19.08	15.76

*Significantly different from baseline control ($p<0.01$).

**Significantly different from control ($p<0.05$).

TABLE 10

MEASUREMENT OF CALCIUM EXCRETION AFTER TAKING TUMS

PRE-DAY NO CALCIUM	33.7 mg/dL
PRE-NIGHT NO CALCIUM	16.7 mg/dL
POST-DAY WITH CALCIUM	66 mg/dL
POST-NIGHT WITH CALCIUM	50 mg/dL

TABLE 11

Evaluation of urine after dietary calcium supplementation.

Group	Epithelial Cell Treatment	Mean Bacteria per Cell	STD. DEV.
Baseline Control	None	0.16	0.37
Control	*E. coli*	*17.38	14.43
Test A	*E. coli* + Pre-day no calcium	15.30	15.97
B	*E. coli* + Pre-night no calcium	12.67	14.25
C	*E. coli* + Post-day with calcium	**23.56	17.40
D	*E. coli* + Post-night with calcium	**28.02	18.52

*Significantly different from baseline control ($p<0.01$).

**Significantly different from control ($p<0.05$).

TABLE 12

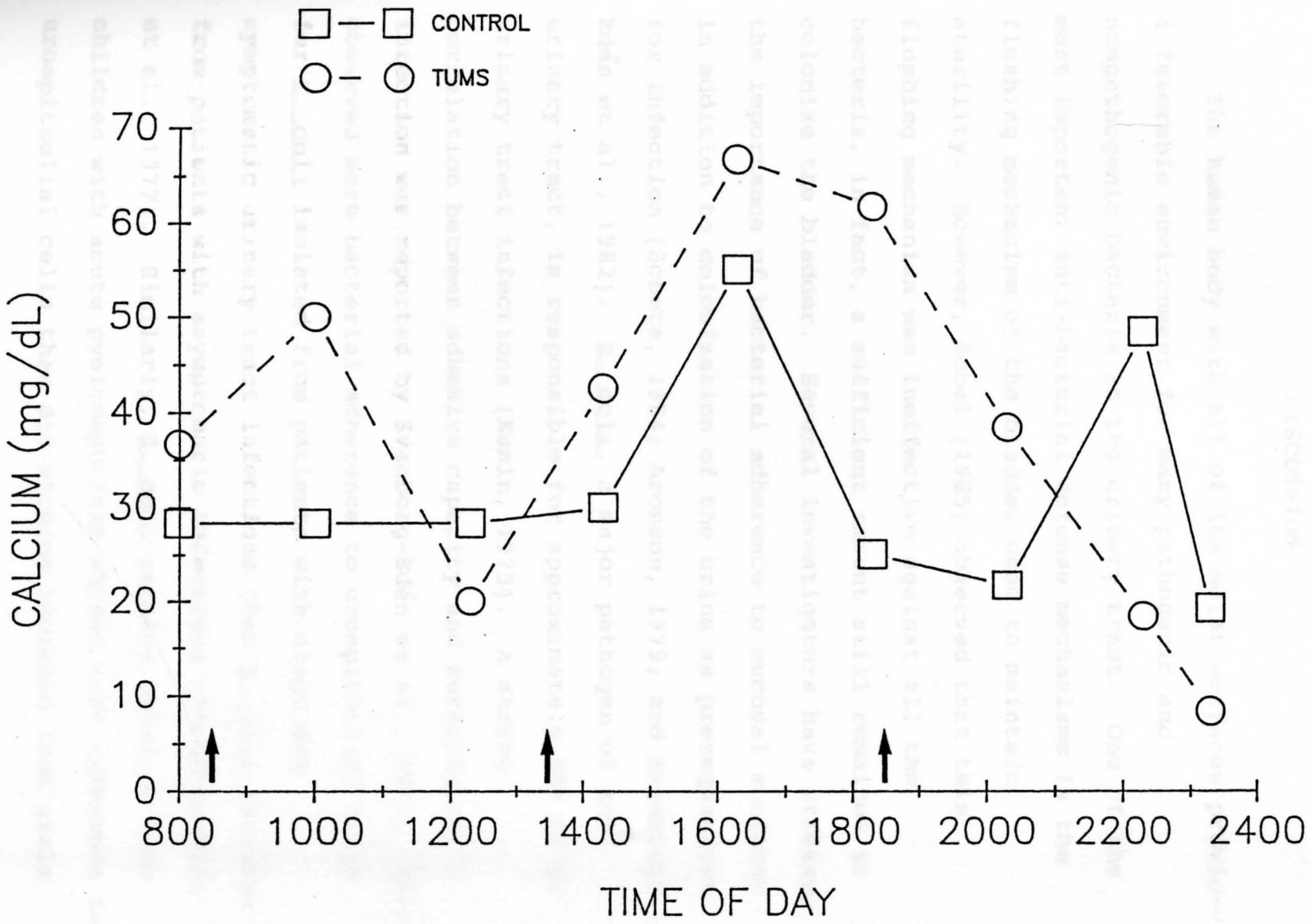
Evaluation of urine after dietary calcium supplementation.

Group	*E. coli* Treatment	Mean Bacteria per Cell	STD. DEV.
Baseline Control	None	0.16	0.37
Control	*E. coli*	*17.38	14.43
Test A	Epi. cells + Pre-day no calcium	16.94	14.95
B	Epi. cells + Pre-night no calcium	13.23	14.34
C	Epi. cells + Post-day with calcium	**28.48	18.76
D	Epi. cells + Post-night with calcium	**23.66	17.82

*Significantly different from baseline control ($p<0.01$).

**Significantly different from control ($p<0.05$).

Fig. 3 Urinary output of calcium after calcium supplementation. Arrows indicate the time that calcium supplements were ingested.


CONTROL
TUMS
CALCIUM (mg/dL)
70
60
50
40
30
20
10
0
800
1000
1200
1400
1600
1800
2000
2200
2400
TIME OF DAY

CHAPTER IV

DISCUSSION

The human body with all of its moist surfaces provides a favorable environment for many pathogenic and nonpathogenic bacteria in the urinary tract. One of the most important anti-bacterial defense mechanisms is the flushing mechanism of the bladder used to maintain sterility. However, Sobel (1985) observed that this flushing mechanism was ineffective against all the bacteria, in fact, a sufficient amount still remained to colonize the bladder. Several investigators have stressed the importance of bacterial adherence to mucosal surfaces in addition to colonization of the urine as prerequisites for infection (Sobota, 1984; Aronson, 1979; and Svanborg-Edén et al., 1982). E. coli, a major pathogen of the urinary tract, is responsible for approximately 80% of all urinary tract infections (Kunin, 1975). A strong correlation between adhesive capacity and severity of infection was reported by Svanborg-Edén et al., 1977. They observed more bacterial adherence to uroepithelial cells for E. coli isolated from patients with diagnosed symptomatic urinary tract infections than E. coli isolated from patients with asymptomatic infections (Svanborg-Edén et al., 1977). Similarly, E. coli strains obtained from children with acute pyelonephritis showed more adherence to uroepithelial cells than did strains isolated from girls

with asymptomatic bacteriuria. This ability of bacteria to adhere to uroepithelial cells is thought to be due to the presence of filamentous structures on the surface of bacteria known as fimbriae (Svanborg-Edén et al., 1978; Pere et al., 1987).

It had been previously demonstrated in this laboratory that calcium ions will interfere with the anti-adherence activity of Tamm-Horsfall glycoprotein, a natural constituent of normal urine (Petruna, 1988). In an attempt to repeat some of these experiments it was observed that the controls that contained calcium alone showed an increase in bacterial adherence. A review of previous experiments, performed by different individuals in the laboratory, confirmed that in some cases when calcium was included in the incubation medium there was an increase in the number of bacteria adhering to uroepithelial cells. This observation led to the present investigation of the effect of calcium on the adherence of bacteria to exfoliated epithelial cells and the related proposition that increased dietary calcium may increase the potential for urinary tract infections. This question is of particular importance currently since it is being suggested that women, who are generally more prone to urinary tract infections than men, are being asked to supplement their diet with increased calcium to counter the effects of osteoporosis.

In the first series of experiments that were performed a clinical isolate of E. coli was incubated in vitro in PBS or sterile urine supplemented with 5 mM or 20 mM calcium. In both cases there was a significant increase in the number of bacteria attaching to exfoliated epithelial cells. Based on our previous data the increase in adhering bacteria after incubation in PBS was expected. The increase in bacterial adherence after incubation in sterile urine was not expected especially in the lower concentration of calcium. Normal urine is known to contain substances that complex calcium ions (Elliot and Eusebio, 1965; Vogel et al., 1984). Through in vitro analysis, Elliot et al., (1965) and Vogel et al., (1984) demonstrated that organic acids present in urine have the potential to complex calcium ions in various solutions. The ability of organic acids to bind calcium was further supported by Pak et al., (1984) in which they observed a 30% increase in the level of urinary citrate and a 40% decrease in calcium after a healthy person ingested 60 mEq/day of potassium citrate. It was anticipated that these factors would reduce the level of added calcium and thus reduce the number of adhering bacteria. In fact the opposite occurred, there was an increase in the number of adhering bacteria after incubation in sterile urine in the lower concentration of calcium. It would thus appear, at least in this instance, that normal urine contains enough calcium to saturate the complexing factors and the addition of even

a low concentration of calcium is sufficient to increase the potential for additional bacterial adherence. This would have particular significance if calcium supplements are used in the diet. It would appear that even the addition of low levels of calcium in the urine may increase the level of bacterial attachment and thus increase the potential for urinary tract infection. Cervera et al. (1987) demonstrated that of 49 children with idiopathic hypercalciuria, a metabolic disorder resulting in an increase in urinary excretion of calcium, the majority had urinary tract infections.

To determine if calcium had to be continually present in the incubation medium to increase bacterial attachment both the bacteria and epithelial cells were preincubated in PBS containing concentrations of calcium ranging from 0.1 mM to 20 mM and then incubated in PBS to allow atttachment. At the higher concentrations, 10 mM and 20 mM, apparently there was enough calcium associated with the surface of either the bacterial or exfoliated epithelial cell to increase attachment. Preincubation of the cells in the lower concentrations of calcium apparently did not provide enough residual calcium when the cells were moved to a non-calcium medium to allow bacterial attachment. At low concentrations calcium has to be continually present to increase bacterial attachment whereas at higher concentrations enough residual calcium remains attached to the cell to increase attachment. The same experiment was

performed substituting sterile urine for the PBS as the preincubation and incubation medium and the results were the same. Thus, the association of calcium with either type of cell seems to be relatively stable since the substances in the urine known to complex calcium did not appear to remove enough calcium from the surface of the cells to measurably decrease bacterial adherence.

The effect of the calcium seems to be relatively non-specific since preincubation of either the bacterial or exfoliated epithelial cells in high concentrations of calcium increased attachment. Svanborg-Edén and Hansson (1978) suggested that fimbriae or substances coappearing with fimbriae may be responsible for the ability of E. coli to adhere to human uroepithelial cells resulting in urinary tract infections. Since the calcium ion contains a positive charge and the cell surfaces are generally negatively charged (Silverblatt et al., 1979; Harber, 1985; and Beachey, 1981) it would appear that calcium simply neutralizes the two surfaces and allows the two cells to approach each other. Once this occurs more specific factors associated with bacterial adherence would then permit attachment.

To determine if any ion could substitute for calcium several additional positive ions were tested in the adherence test. The addition of monovalent sodium and potassium ions to the incubation medium had no effect on adherence suggesting that not just any positive ion will

increase the attachment of the bacteria to exfoliated epithelial cells. Magnesium, a divalent cation, also did not increase bacterial attachment, in fact in the presence of this ion there was a significant decrease in adherence. Thus it would appear that there is at least some specificity associated with the increase in adherence observed in the presence of excess calcium.

Since the in vitro work described above suggested that excess calcium enhances bacterial adherence, an evaluation of increased dietary calcium was undertaken to determine if this would lead to an increased excretion of calcium in the urine and an attendant increase in bacterial attachment. TUMS, a popular antacid, has been suggested as a calcium supplement for women to protect against osteoporosis. A typical regimen consists of 3 tablets per day (Goulder and Lutwak, 1988). In this study this regimen was followed and 24 hour urine was collected prior to and after dietary supplementation with calcium. The excretion of calcium in the urine after ingestion of the TUMS approximately doubled. This is equivalent to the concentration that increased bacterial attachment in vitro. Incubation of either the bacteria or the exfoliated epithelial cells in urine before ingestion of the calcium supplement showed no increase in adherence, whereas urine collected after dietary supplementation showed a significant increase in bacterial attachment.

Based on the results of the previous experiment, urine was collected every two hours to determine the specific area of time in which the level of urinary calcium was the highest. The results showed a direct correlation between the time of TUMS consumption and the urinary output of calcium. For every tablet that was consumed there was an increase in the level of calcium in the urine as compared to the control in which no TUMS were ingested. The greatest amount of calcium detected in the urine after the ingestion of TUMS was observed at 4:30 pm. This observation suggests that about late afternoon enough calcium may be present in the urine to enhance bacterial adherence to exfoliated epithelial cells and possibly increase the potential for urinary tract infection.

In summary, bacterial adherence appears to be a significant factor for urinary tract infections. Since increased calcium has been shown to increase bacterial adherence *in vitro*, and calcium supplements increase the level of calcium in the urine so that this urine increases bacterial attachment, it would appear that increased dietary calcium has the potential to enhance the possibility of urinary tract infection.

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