Scanning Microscopy

Volume 9 | Number 1 Article 5

1-10-1995

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Scanning Microscopy, Vol. 9, No. 1, 1995 (Pages 89-101) 0891-7035/95$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

EXPERIMENTAL CALCIUM OXALATE NEPHROLITHIASIS AND THE FORMATION OF HUMAN URINARY STONES

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(Received for publication August 20, 1994 and in revised form January 10, 1995)

Abstract

Calcium oxalate nephrolithiasis in rats requires induction of hyperoxaluria which results in increased urinary calcium oxalate supersaturation. As a result of low to mild chronic hyperoxaluria, calcium oxalate crystals deposit first in the papillary collecting ducts. Crystal deposition in the kidneys is preceded by calcium oxalate crystalluria and starts with the retention of aggregated calcium oxalate crystals in the renal tubules. Retained crystals move from the tubules to the interstitium, and in the process, become anchored to the tubular basement membrane. Crystal aggregates present in the superficial peripheral collecting ducts of the renal papillae ulcerate through to the papillary surface and grow into the stones.

Key words: Calcium oxalate, nephrolithiasis, urolithiasis, hyperoxaluria, crystallization, crystalluria.

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Introduction

Urinary stone formation or urolithiasis is a common human urological disorder and has been recognized as a health problem since the beginning of recorded history. Many *in vitro* and *in vivo* models have been developed to understand the mechanisms involved in the formation of urinary stones and to ascertain the effects of various therapeutic agents and protocols on development and progression of the disease. Calcium oxalate stones are most common. As a result, calcium oxalate urolithiasis has been studied in greater detail. The rat is the most frequently used animal in many such studies and induction of hyperoxaluria is the most common means to promote calcium oxalate urolithiasis. Various approaches applied in this regard were reviewed in 1985 (Khan and Hackett, 1985), and therefore, will not be considered here. Only the information published since that review and the knowledge obtained by using the rat models will be discussed in this paper. Pathogenesis of urolithiasis involves crystal nucleation, growth, aggregation and retention of crystals within the urinary system. A variety of modulators control these processes by promoting, inhibiting or modifying them. The final event in stone formation is the evolution of retained crystals into urinary stones, a process which has not yet been dealt with experimentally.

Crystal Nucleation

Rat models have provided a wealth of information about how and where crystals of calcium oxalate are formed in the kidneys. Like humans, there is no spontaneous production of urinary stones by normal rats. Induction of hyperoxaluria is essential. Thus, rat models are similar to the human condition because in humans too, hyperoxaluria is regarded as one of the most common causes of idiopathic calcium oxalate urolithiasis (Robertson and Peacock, 1985; Coe *et al.*, 1992; Khan 1992). In male Sprague-Dawley rats, acute hyperoxaluria or moderate to high grade chronic hyperoxaluria, the latter being a condition somewhat similar to primary

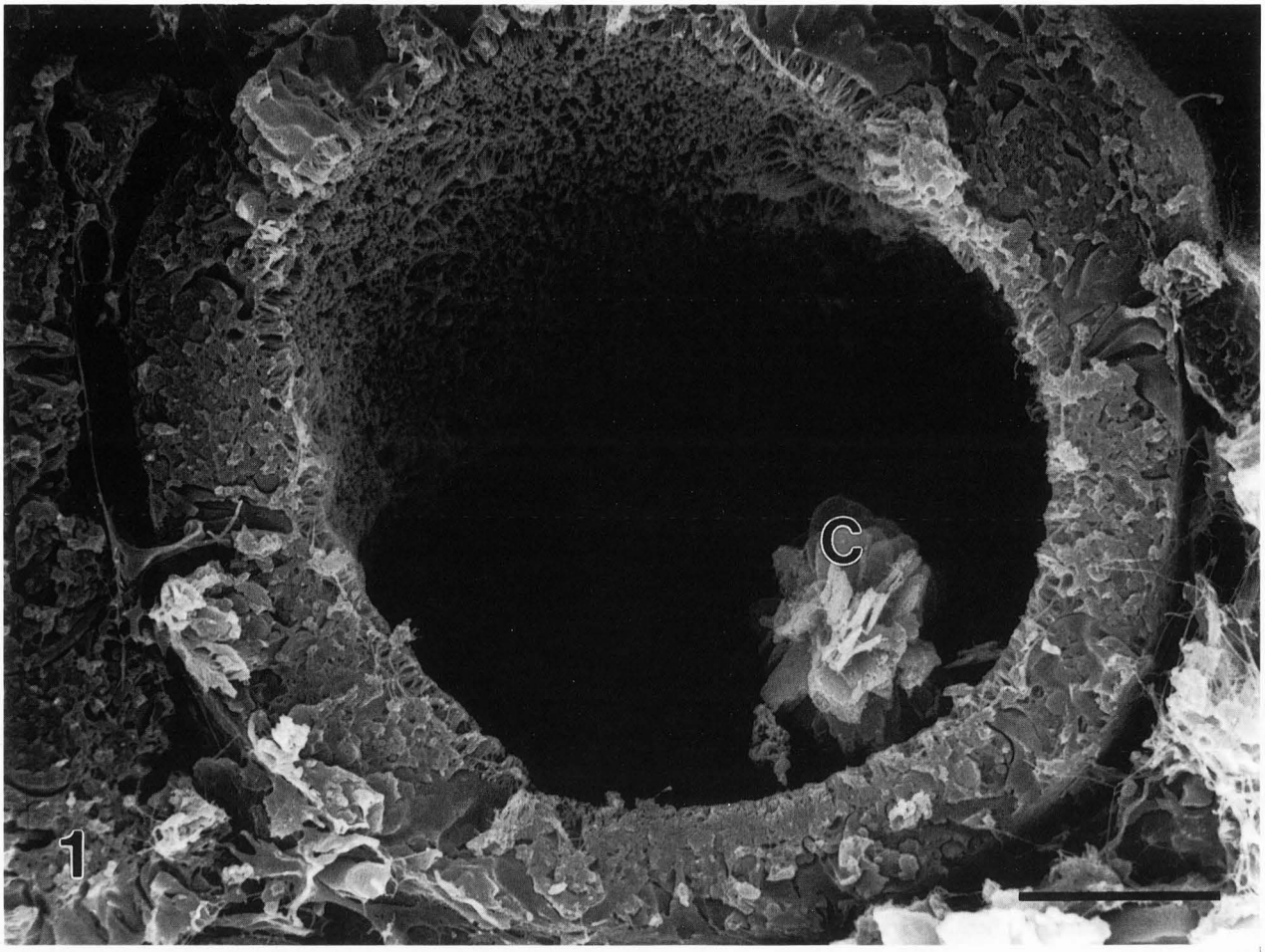

Figure 1. Calcium oxalate (CaOx) monohydrate crystals (C) present in the lumen of the renal proximal tubule of a male rat, 1 hour after sodium oxalate injection at the dose of 7 mg/ 100 g rat body weight. Bar = 5 μm.

hyperoxaluria in humans, results in initial calcium oxalate crystal deposition in the proximal segments of the renal tubules, while a low grade chronic hyperoxaluria, a condition probably similar to most hyperoxaluric stone formers, starts the deposition of crystals in the distal segments of the renal tubules (Khan, 1991). Obviously, in the first situation, urine becomes highly supersaturated with respect to calcium oxalate much earlier in the nephron, while in the second, urine becomes highly supersaturated with respect to calcium oxalate when it reaches the collecting duct system. This may be one of the reasons that the renal papilla is the most common site of human urinary stone formation.

Morphological studies have demonstrated that crystals of calcium oxalate first appear in the renal tubular lumina (Fig. 1) (Khan *et al.*, 1979, 1982). Crystals that are left behind are later found in the interstitium (Fig. 2) or the tubular epithelial cells (Khan *et al.*, 1992a). These findings suggested that calcium oxalate crystals are initially formed in the lumen of renal tubules from where most of them are flushed out with the urine. The rest of the crystals migrate to the interstitium. A recent study has provided further evidence for this migration from the tubular lumen to the interstitium (de Bruijn *et al.*, 1994). Intra-tubular crystals were produced in rat kidneys by administering a crystal-inducing regimen which involved mixing ethylene glycol and ammonium chloride in the drinking water. After nine days of such treatment, the rats were given regular water and diet for the next four days to flush out the free luminal crystals. The rats were divided into two groups. Those of group 1 were sacrificed at this time and their kidneys processed for microscopic examination. Rats of group 2 were then given a sublithogenic challenge for 12 or 30 days. Light microscopic examination of plastic embedded sections of the kidneys from the first group of rats showed that crystals were present: (1) as aggregates blocking the tubular lumina; (2) attached to the epithelial cell surface;

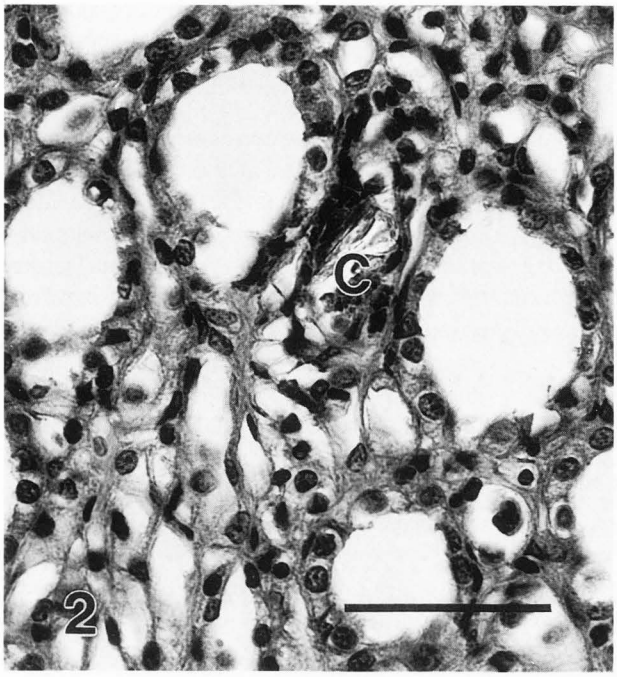

Figure 2. Birefringent crystals of CaOx monohydrate (C) present in the renal papillary interstitium, 6 hours after sodium oxalate injection. Bar = 50 μm.

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and (3) between or under the epithelial cells. Even after 30 days, kidneys of group 2 rats contained crystals, but these crystals were now present in the renal interstitium. These observations indicate that crystals retained inside the renal tubular lumina are overgrown by several epithelial cells, and then, are pushed out of the tubules into the interstitium.

Presence of crystals in the cells may either indicate endocytosis or their formation inside the cells (Boeve *et al.*, 1993). When exposed to preformed crystals, cultured epithelial and other types of cells have been shown to endocytose a variety of crystal types (Emmerson *et al.*, 1990), including calcium oxalate (Lieske *et al.*, 1992; Lieske and Toback, 1993; Hackett *et al.*, 1994). A similar phenomenon is possible during nephrolithiasis. In most of the animal model studies where the time course of crystal formation and retention in the kidneys is known, appearance of crystals within the epithelial cells happens after the crystals have already been seen inside the tubular lumina (Khan *et al.*, 1992a). In many cases, intracellular crystals are observed after large crystalline deposits have already been deposited within the renal tubules. Crystal deposition within the renal tubules is invariably associated with cellular damage. Damage to the cell is initially indicated by distortion of the brush border membrane (Fig. 3), clubbing of the microvilli and formation of large blebs (Khan and Hackett, 1985;

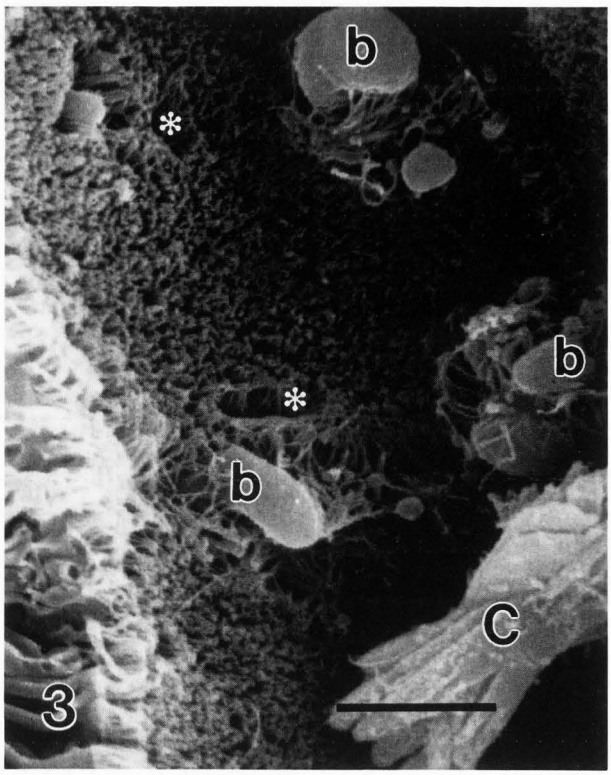


Figure 3. Formation of blebs (b) and loss of brush border membrane (*) in the presence of CaOx monohydrate crystals (C) 1 hour after sodium oxalate injection. Bar = 5 μm.

Boeve *et al.*, 1993). Increased lipid peroxidation has also been demonstrated in the kidneys of nephrolithic rats (Rengaraju and Selvam, 1989; Ravichandran and Selvam, 1990). All these changes indicate membrane damage. Once the cell membrane is damaged, it can no longer maintain its homeostatic function; as a result, calcium and other ions may freely move in and out, resulting in an increased supersaturation within the confines of the cells, and eventually, causing the formation of crystals within the cells.

Rat studies of calcium oxalate urolithiasis demonstrate that crystalluria and nephrolithiasis occur at an approximate relative supersaturation of 20 (Khan and Hackett, 1987a; Khan *et al.*, 1992b), which is not high enough for homogeneous nucleation of calcium oxalate crystals (Finlayson, 1978). Thus, it is most likely that crystal nucleation in rat urine is heterogeneous. What biological or crystalline materials can act as the substrate for crystal nucleation in nephrolithiasis? It has been suggested that calcium phosphate crystals may act as nucleators of calcium oxalate, since calcium phosphate is the most common crystal found in human urine and urinary stones (Murphy and Pyrah, 1962; Werness *et al.*,

1981) and can cause nucleation of calcium oxalate *in vitro* from a metastable solution of calcium oxalate (Meyer *et al.*, 1975). Our extensive studies of calcium oxalate nephrolithiasis in rats have failed to demonstrate any calcium phosphate in association with deposits of calcium oxalate in the kidneys.

We tested the hypothesis that renal deposits of calcium phosphate can provoke precipitation of calcium oxalate within the renal tubules (Khan and Glenton, 1995). We induced calcium phosphate renal deposits followed by mild hyperoxaluria. In one experiment, both male and female rats were first given a calcium phosphate-inducing diet, the so-called AIN 76 diet, and then, put on a hyperoxaluria-inducing protocol involving mixing of ethylene glycol in the drinking water. Female rats responded to the calcium phosphate-inducing diet by generating calcium phosphate crystal deposits in renal tubules at the cortico-medullary junction. Male rats responded to the hyperoxaluric challenge by producing calcium oxalate crystals in the collecting ducts of the renal papillae. Small deposits of calcium oxalate were seen in kidneys of the female rats on hyperoxaluria-inducing protocol. Male rats on calcium phosphate-inducing diet did not produce any calcific deposits in their kidneys. In another experiment, a calcium phosphate-inducing diet and hyperoxaluric challenge were simultaneously delivered to female rats. This resulted in calcium phosphate and calcium oxalate crystalluria as well as nephrolithiasis, but calcium oxalate crystals were not observed in association with calcium phosphate crystals. Results of these experiments suggest that calcium phosphate is not necessary for calcium oxalate nephrolithiasis.

Then what is the heterogeneous nucleator of calcium oxalate crystals? Urinary as well as renal calcium oxalate crystals induced in rats are almost always found associated with cellular degradation products, including their membranous components (Khan and Hackett, 1985). Membranous cellular degradation products and their constituent lipids are an important part of the matrix of human urinary stones (Khan *et al.*, 1988). Both the lipids isolated from human urinary stones and the membrane vesicles obtained from renal proximal tubular brush border have been shown to induce crystallization of calcium oxalate from a metastable solution (Khan *et al.*, 1988, 1989, 1993). Moreover, thousands of epithelial cells are released in the urine by mammalian kidneys. Human kidneys release approximately 70,000 cells/hour (Prescott, 1966). Thus, mammalian urine is replete with cellular degradation products which can nucleate calcium oxalate crystals and do not require any other type of heterogeneous nucleators. This abundance of nucleators in the urine may be the reason why mammalian urine contains such a variety of crystallization inhibitors (Robertson and Peacock, 1985; Coe *et al.*, 1992; Khan, 1992).

Mammalian urine contains both inhibitors and promoters of crystallization (Khan, 1992). In normal individuals, inhibitors may have the upper hand, but in stone formers, urine is either less inhibitory or becomes less inhibitory during stone forming episodes. Alternatively, the promotory potential of urine may increase by virtue of an increase in heterogeneous nucleators. We decided to test this hypothesis by increasing the membranous cellular degradation products as heterogeneous nucleators in the urine (Hackett *et al.*, 1990). Membranuria of renal proximal tubular origin was induced by administration of gentamicin sulphate to male Sprague-Dawley rats. A low grade hyperoxaluria was produced by ethylene glycol administration. Hyperoxaluria or gentamicin treatment alone did not cause the formation of crystals in the urine, but simultaneous production of membranuria and hyperoxaluria resulted in calcium oxalate crystalluria. Crystals were formed in association with the membranous substances in the urine. These observations were later confirmed when a higher grade hyperoxaluria in association with gentamicin-induced membranuria resulted in 63% of rats with calcium oxalate nephrolithiasis (Kumar *et al.*, 1991). Hyperoxaluria alone induced nephrolithiasis in only 6% of the treated rats.

Sex Hormones and Nephrolithiasis

Sex is a well known risk factor for the formation of urinary stones. Idiopathic calcific stone disease is two to three times more common in men than in women (Robertson and Peacock, 1985). Kidney stones found in men are normally calcium oxalate while those in females are usually calcium phosphate (Otnes, 1980). Reasons for these differences between sexes are still not clear. The lower rate of calcific nephrolithiasis in women is suggested by some to be a result of higher urinary excretion of citrate (Tiselius, 1985), and by others, an effect of lower urinary excretion of calcium and oxalate (Robertson *et al.*, 1978). Finlayson (1974) postulated that lower testosterone levels might cause lower excretion of oxalate and contribute to some of the protection against calcium oxalate stone formation in women. Results of studies involving rat models of nephrolithiasis have provided some explanation for this interesting phenomenon.

Spontaneous deposition of calcium phosphate in the kidneys of rats on semi-purified diets, like AIN-76, has been reported by many investigators and the incidence of lesion formation is consistently higher in females than males (Geary and Cousins, 1969; Nguyen and Woodard, 1980). Calcification starts intraluminally at the cortico-medullary junction spreading into the medulla. Estro-

gen, in addition to the dietary levels of calcium, phosphorus and magnesium has been determined to play a significant role in the development of this disease. Ovariectomy resulted in cessation of calcification, while replacement therapy with estrogen following gonadectomy resulted in calcium phosphate nephrolithiasis in both male and female rats (Geary and Cousins, 1969).

Administration of 1% ethylene glycol in drinking water for 4 weeks produced calcium oxalate nephrolithiasis in 3/13 males but 0/12 females (Lyon *et al.*, 1966). Urinary acidification increased the incidence to 5/6 males and 1/9 females. These observations demonstrated an association between gender and urolithiasis but did not provide a justification for the phenomenon. In an attempt to determine the role of testosterone, 0.5% ethylene glycol was used to induce calcium oxalate nephrolithiasis in male and female, normal and gonadectomized rats (Lee *et al*, 1992). Low level calcium oxalate crystal deposition was found in all rats but in normal males, 5/7 produced kidney stones and 4/7 had massive calcium oxalate crystal deposition. Only 1/7 castrated males produced kidney stones and none of them had massive calcium oxalate crystal deposition in their kidneys. None of the female rats, normal or castrated, produced any kidney stones or massive crystal deposition in their kidneys. As a result of the study, it was suggested that testosterone plays a determinant role in the pathogenesis of urolithiasis.

As indicated earlier, we recently studied calcium phosphate and calcium oxalate nephrolithiasis in both male and female rats (Khan and Glenton, 1995). Calcium phosphate deposition was induced by feeding a semi-purified diet, the AIN-76, and calcium oxalate, by administration of ethylene glycol in association with urinary acidification. Rats of the same strain and same age and similar weight were used. Calcium phosphate deposition was confined to the cortico-medullary junction as has been demonstrated by other investigators and calcium oxalate was mostly localized to renal papillae and fornices. All rats of both sexes receiving ethylene glycol had calcium oxalate deposits in their kidneys but only male rats had massive crystal deposition and crystals at the renal papillary tips. Only female rats produced calcium phosphate deposits on the AIN diet. These results are similar to those of earlier investigators discussed above. But both male and female rats had similar relative supersaturations for both calcium oxalate and calcium phosphate. Lee *et al.* (1992) have also shown that gonadectomy did not significantly influence the urinary biochemistry. Then, why did male and female rats behave differently when it came to nephrolithiasis? Nephrolithiasis involves crystal formation and retention (Finlayson and Reid, 1978). The latter can be facilitated by crystal aggregation (Kok and Khan, 1994). Perhaps, there are some differences in urinary inhibitors of crystal aggregation between males and females.

Crystal Growth and Aggregation

It is generally agreed that stone forming humans excrete larger and more aggregated crystals than the non-stone formers (Robertson *et al.*, 1971). No specific study has been done to examine crystal growth and aggregation in rat models of urolithiasis and no quantitative data are available. However, morphological examination of crystals produced by nephrolithic rats has shown that first-formed crystals are generally small and single, and easily pass through the nephron and clear the kidneys. In later stages of the disease, both excreted crystals, as well as those retained inside the nephron, are larger and aggregated (Khan and Hackett, 1991).

Crystallization Modulators

There are two major categories of crystallization modulators present in human urine (Robertson and Peacock, 1985; Khan, 1992). One consists of citrate (Meyer and Smith, 1975; Antinozzi *et al.*, 1992) pyrophosphate (Fleisch and Bisaz, 1962) and magnesium (Desmars and Tawashi, 1973) and the other of urinary macromolecules, Tamm-Horsfall protein (THP) (Hess, 1991), osteopontin (OP, uropontin) (Shiraga *et al.*, 1992), nephrocalcin (NC) (Nakagawa *et al.*, 1984), uronic acid rich protein (UAP) (Atmani *et al.*, 1993; Atmani and Khan, 1995), and inter-α-trypsin inhibitor (Sorensen *et al.*, 1990). Magnesium and citrate form soluble complexes with oxalate and calcium, respectively (Robertson and Peacock, 1985). Citrate also binds to the surface of calcium oxalate crystals, interferes with their growth and modifies their morphology (Antinozzi *et al.*, 1992; Shirane and Kagawa 1993). Magnesium has been extensively studied in animal models of calcium oxalate urolithiasis and it has been shown that magnesium administration in the form of alkalinizing salts causes a decrease in calcium oxalate crystal deposition in the kidneys of hyperoxaluric rats (Ogawa *et al.*, 1990; Su *et al.*, 1991; Khan *et al.*, 1992b). Magnesium reduces the amount of oxalate excreted in the urine, thereby resulting in a reduced urinary calcium oxalate relative supersaturation.

Much of the information about THP, OP (uropontin), NC, and UAP, including their normal renal distribution has been obtained from studies of rat kidneys by using immunohistological techniques and *in situ* hybridization. THP is localized to the thick ascending limb of the loop of Henle (Bachmann *et al.*, 1990), NC to the proximal tubule as well as thick ascending limb of the loop of Henle (Nakagawa *et al.*, 1984; Srivongs *et al.*,

1989), while OP to the collecting duct system (Lopez *et al.*, 1993). However, all the tubules do not stain for the OP. Uronic acid rich protein has also been isolated from both rat and human urine (Atmani *et al.*, 1993; Atmani and Khan, 1995). Most of the information about their roles in calcium oxalate crystallization comes from *in vitro* studies, mainly performed in inorganic solutions. OP, NC and UAP are potent inhibitors of calcium oxalate crystallization while the role of THP is controversial (Hess, 1991). Depending upon pH, glycosylation, self-aggregation and concentration of other ions in the solution, THP can be a promotor or inhibitor of crystallization. Results of one study carried out in human urine demonstrated that the effect of THP on calcium oxalate crystallization depends upon the methodology used to asses it (Grover *et al.*, 1990). THP promoted calcium oxalate crystallization induced by evaporation of urine but inhibited growth and aggregation of calcium oxalate crystals induced by addition of an oxalate load.

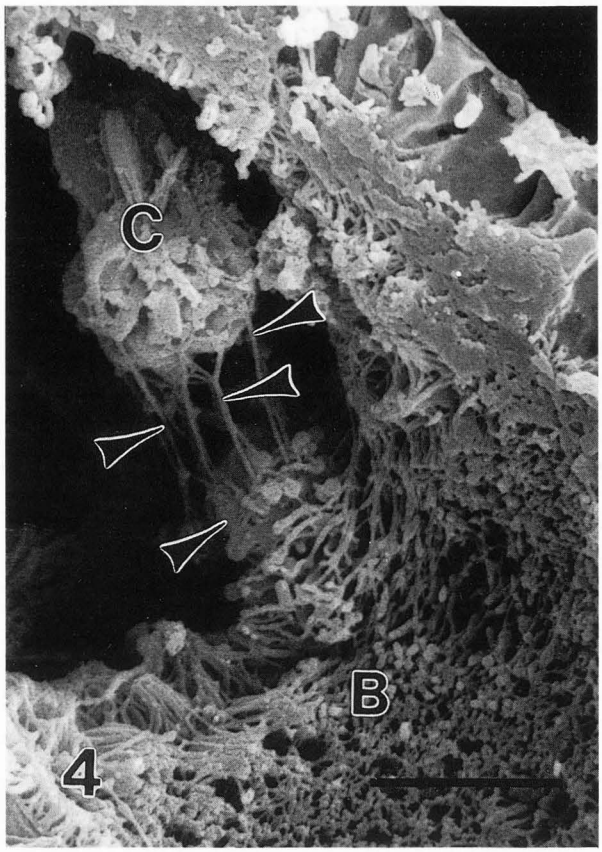
Recently, the distribution of THP and OP was studied in rat kidneys with calcium oxalate nephrolithiasis (Gokhale *et al.*, 1994 and unpublished personal observations). Mild chronic hyperoxaluria was induced in male Sprague-Dawley rats, which resulted in calcium oxalate crystal deposition in renal papillae. Kidney sections were stained for both THP and OP using polyclonal antibodies. Crystal deposits were surrounded by THP as well as OP. Epithelial cells around the crystal deposits were also positive for THP as well as OP. Both THP and OP are generally absent from the papillary ducts, their lumina as well as the epithelial cells. Presence of THP and OP in association with the crystals may be accidental due to the tubular blockage by the crystals or indicate THP and OP involvement in crystal deposition. Transmission electron microscopic examination of the demineralized calcium oxalate crystals stained for OP demonstrated the incorporation of the protein into the organic matrix of crystals (McKee and Khan, 1994). This indicates a much closer affiliation between calcium oxalate crystal formation and OP.

Ryall and associates isolated a matrix protein from calcium oxalate crystals newly generated in the fresh whole normal human urine (Doyle *et al.*, 1991). They aptly named the protein, crystal matrix protein or CMP. CMP was found to be a potent inhibitor of calcium oxalate crystallization and was localized to the distal convoluted tubule and the thick ascending limb of the loop of Henle using a polyclonal antibody raised against human CMP (Stapleton *et al.*, 1993b). Recently, 81.8% sequence identity was revealed between 11 N-terminal amino acids of CMP and N-terminus of human prothrombin. The relationship between human CMP and prothrombin was confirmed by demonstrating the reaction between an antibody to human prothrombin and CMP western blots (Stapleton *et al.*, 1993a). CMP or prothrombin has not yet been studied in human stone formers or animal models of nephrolithiasis.

Crystal Retention

Crystal retention is indispensable to the development of kidney stones, and rat models have provided valuable information about the mechanisms involved in this process. Crystal size can play an important role, and thus, any mechanism, that can result in mass accretion by the crystal deposits, can regulate the process of crystal retention. Taking into account the rate of crystal growth, dimension of the renal tubules and the time it takes the urine to pass through the nephron, Finlayson and Reid (1978) concluded that crystals of calcium oxalate can not grow fast enough to be retained within the renal tubules because of their size alone. They concluded that it is necessary for the crystals to attach to the tubular epithelium for retention within the kidneys. We recently revisited this issue, calculated the rate of mass accretion by crystal deposits taking into account the process of crystal aggregation, and found that crystal aggregates can become large enough to block the renal tubules, and thus, be retained solely because of the aggregate size (Kok and Khan, 1994). Animal studies have demonstrated that as the disease progresses, more aggregated crystals are formed and aggregates are generally large enough to be retained without attachment to the renal tubules. Often, the crystals deposit at sites where the luminal diameter of the tubules narrows, e.g., at the proximal tubule/loop of Henle junction or at the papillary base where renal tubules bend (Khan and Hackett, 1991). It is also interesting to point out that the openings of the ducts of Bellini into the renal pelvis are not circular, but slit-like, and are much narrower than the luminal diameter of the ducts of Bellini.

Crystals have also been found to be retained inside the tubules by attachment to the renal epithelial cell surface (Fig. 4) as well as epithelial basement membrane (Khan *et al.*, 1982; Khan and Hackett, 1991). It has also been demonstrated that calcium oxalate crystals adhere to the surface of injured rat renal papillary epithelial cells in primary culture (Riese *et al.*, 1992a,b). Calcium oxalate crystal deposition in the renal tubules is associated with injury to the epithelium which often results in detachment of the cells from its basement membrane. In an experiment where acute hyperoxaluria was induced by intraperitoneal injection of sodium oxalate, crystal attachment to the basement membrane was seen hours after the challenge (Khan *et al.*, 1982) suggesting that, most probably, it is not the result of crystal nucleation by the basement membrane constituents but rather a phenomenon of crystal attachment. As discussed earlier,



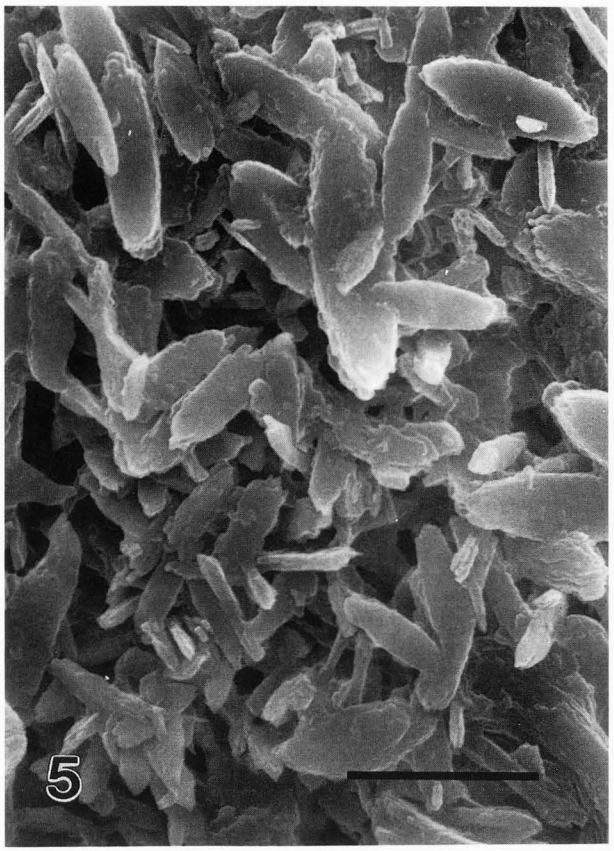


Figure 4 (at left). Crystals of CaOx monohydrate (C) are attached to the brush border (B) of a renal proximal tubule by tangling with microvillous projections (arrowheads). Bar = 5 μm.

Figure 5 (at right). Agglomerate of CaOx monohydrate crystals present in the bladder urine of a rat receiving 0.75% ethylene glycol in drinking water. Crystals are associated with an amorphous matrix material. When similar crystals were examined by transmission electron microscopy, the amorphous substance surrounding the crystals was identified as cellular degradation products. Bar = 5 μm.

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crystals retained inside the tubular lumen can migrate to the interstitium.

It has been argued that crystals actually form in the renal papillary interstitium (Hautmann and Osswald, 1983), since most of the oxalate, particularly that in the renal papilla, is present in the interstitium, and the papilla has the highest concentration of calcium and oxalate within the kidney (Wright and Hodgkinson, 1972). If crystals have already formed in the interstitium then retention will not be an issue. However, all morphological studies of nephrolithiasis, some of them discussed above, have clearly shown that initial crystal formation starts in the tubular lumen. Interstitial crystals are seen much later during nephrolithiasis. In addition, once crystals move into the interstitium, they do not seem to grow. They actually seem to disappear (Khan *et al.*, 1992a).

Crystals are also seen inside the epithelial cells and this could indicate another means of retention, however, such cells normally appear damaged and may be sloughed off and take the crystals with them. It has, however, recently been shown that renal epithelial cells in culture can function normally, even multiply, after endocytosing the crystals (Lieske *et al.*, 1992; Lieske and Toback, 1993). Thus, healthy cells may also contain crystals, but these crystals will not be available for further growth and may not be available for stone formation. Moreover, such endocytosis may represent a defensive mechanism against injury-causing calcium oxalate crystals.

It has been proposed that crystals of calcium oxalate may form subepithelially on the renal papillary surface, resulting in spontaneous retention (Chandhoke and Hruska, 1993). The crystals then ulcerate to the surface where they are exposed to the pelvic urine and provide a nidus for the development of urinary stones. The papillary surface is a common site for the formation of

urinary stones in humans.

Renal Injury and Nephrolithiasis

Human stone forming patients generally display abnormal urinary chemistry and some form of functional or structural tubular abnormalities and/or tubular damage (Jaeger *et al.*, 1986). This aspect of kidney stone formation has been extensively studied in rat models (Khan *et al.*, 1979, 1982, 1992a; Khan and Hackett, 1985; Kumar *et al.*, 1991). It has been found that calcium oxalate crystallization results in both covert and overt damage to the renal tubular epithelium. The extent of the damage appears to be related to the magnitude of hyperoxaluria. A low grade hyperoxaluria, which caused no crystal deposition in the kidneys, still resulted in damage to the proximal tubular epithelial cells as evidenced by increased enzymuria (Khan *et al.*, 1989). This is important information, since most human stone formers have only a mild hyperoxaluria and also display enzymuria of proximal tubular origin (Baggio *et al.*, 1983). In addition, recent studies using cultured epithelial cells have also shown that the oxalate ion itself can be damaging to the cells (Hackett *et al.*, 1994). It has already been established that cells exposed to many types of crystals, including calcium oxalate, are invariably injured. Injury results in cell sloughing and membranuria. Both aggregated, as well as single crystals of calcium oxalate present in the kidneys or the urine (Fig. 5), are always seen associated with cellular membranes. Sloughed cellular membranes may become involved in both crystal nucleation and aggregation (Khan and Hackett, 1987b). Cell sloughing also exposes the basement membrane which can be a likely site for crystal attachment and retention and thus initiation of stone formation.

Many macromolecules involved in calcium oxalate crystallization are produced by renal tubular epithelial cells, particularly the epithelium lining the proximal tubules and the loop of Henle (Coe *et al.*, 1991). Any damage to these cells may interfere with the production of crystallization inhibitory macromolecules. Urine of rats treated with gentamicin sulphate, which is specifically toxic to the proximal tubular epithelial cells of the kidneys, has already been shown less inhibitory to calcium oxalate crystallization processes (Finlayson *et al.*, 1989).

Development of Urinary Stones

Crystal retention is essential for the development of stone disease, but how do the retained crystals develop into kidney stones? No studies, animal or otherwise, have been performed to specifically study the transition from retained crystals to kidney stones. However, a number of observations have been made that can assist us in developing a probable scenario. It has been demonstrated that induction of acute hyperoxaluria results in the development of an instantaneous calcium oxalate nephrolithiasis in male rats (Khan *et al.*, 1979, 1992a). Crystals are seen in the kidneys up to seven days after the challenge. They are first seen in the tubular lumen and later, by day seven, in the interstitium as well as inside the cells. Thus, crystals move from the tubular lumen to the interstitium. Another, more recent study of chronic hyperoxaluria in male rats has similarly demonstrated crystal movement from the lumen to the interstitium (de Bruijn *et al.*, 1994). Both studies have also shown that the number of retained crystals appears to decrease with time. In addition, we have recently observed interstitial crystals surrounded by cells which are generally involved in the inflammatory processes (unpublished personal observations).

Crystals are retained at many sites in the kidneys, the most common being the cortico-medullary junction (Khan and Hackett, 1991). Kidney stones, however, develop on papillary surfaces (Figs. 6a and 6b) in the renal calyx and fornix. What are the reasons for stone development in association with the renal papillae and at these particular sites? One reason may be that the renal papilla has the highest concentration of calcium and oxalate relative to the cortex and medulla (Wright and Hodgkinson, 1972; Hautmann and Osswald, 1983). Other reasons, as discussed in earlier paragraphs, may be related to the anatomy and histology of the papilla (Fig. 6c) which make it possible for the crystals to be retained at these sites. It has already been discussed above that, with passage of time, luminal crystals move out of the tubule and into the interstitium. Apparently, the crystals retained in peripheral collecting ducts and those retained in more central collecting ducts follow different paths. The crystals retained in more central ducts move into the renal interstitium (Fig. 6a) towards the interior of the kidney. Once in the interstitium, they are not exposed to the urine and hence to the urinary calcium and oxalate ions. Thus, they are unable to grow. Moreover, now they may be subjected to the body's inflammatory process and be finally destroyed. On the other hand, when crystals are retained in the peripheral collecting ducts, or in ducts at the papillary tips or base, they can ulcerate to the papillary surface (Figs. 6a and 6b). Once out on the surface, they are continuously bathed in pelvic urine and urinary calcium and oxalate ions, and can grow indefinitely. Thus, it is clear that for the development of kidney stones, not only must the crystals be retained in the kidneys, but they should be located in the tubules from where they can ulcerate to the surface, be exposed to urine, and be available for further growth.

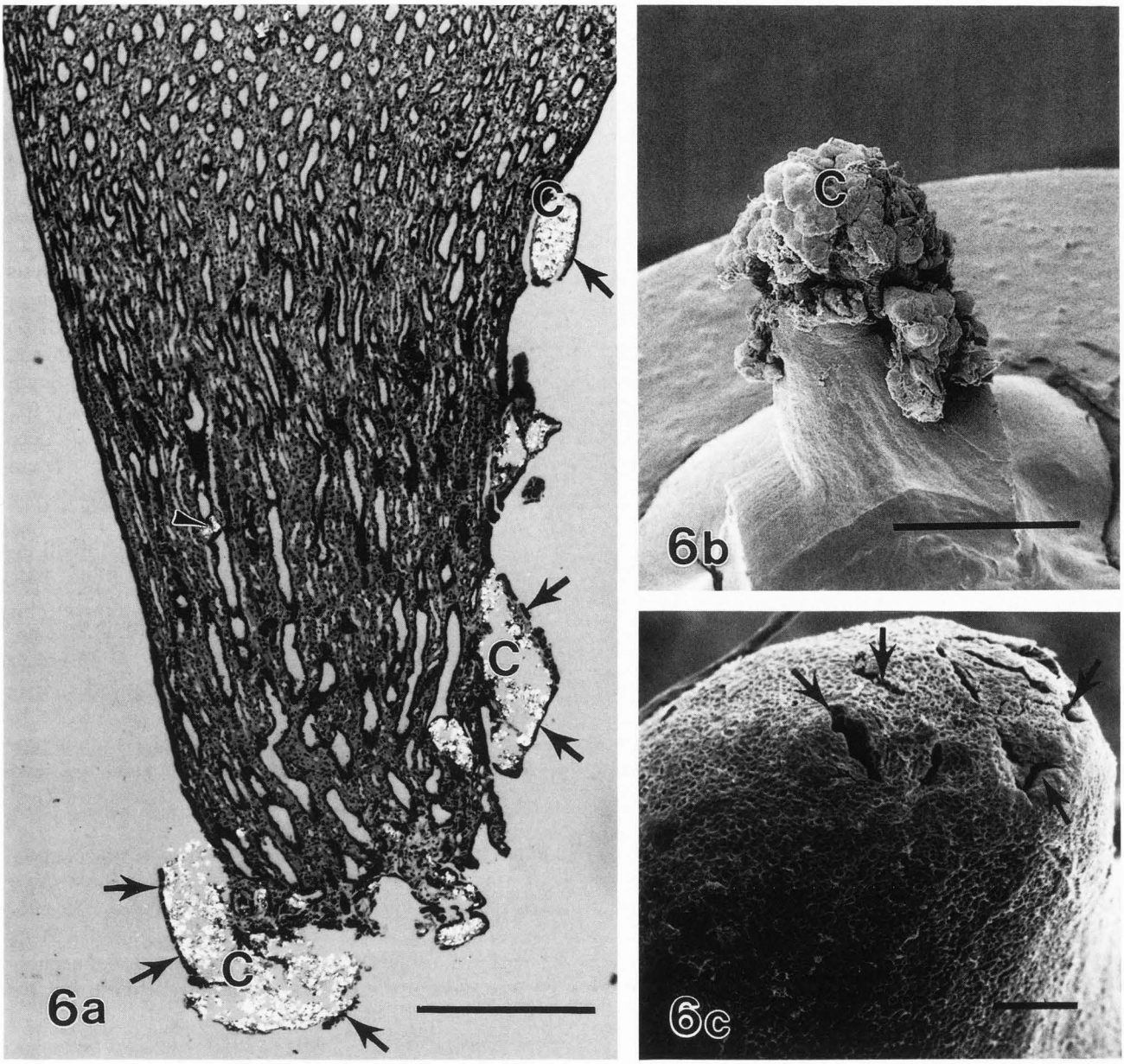

Figure 6. (**a**). Light micrograph of a median section through the renal papilla of a rat receiving 0.75% ethylene glycol in drinking water. Papillary tip and sides are covered with CaOx monohydrate crystal deposits (C). Section shows an epithelial covering (arrows) over the deposits indicating that crystals originated inside the papilla and near the surface. Some crystals, present in the papillary interior, are located in the interstitium (arrowhead). Bar = 500 μm. (**b**). Scanning electron micrograph of the papilla from a rat with similar treatment as rat in Figure 6a. A large CaOx stone (C) is present at the papillary tip. Bar = 1000 μm. (**c**). Papillary tip of a normal rat on normal water and diet showing openings of the ducts of Bellini (arrows). Even though the openings are large lengthwise, they are slit-like with narrow width which will impede the movement of big crystal aggregates. Bar = 100 μm.

Acknowledgements

This work was supported in part by NIH grants numbers PO1 DK 20586 and RO1 DK 41434.

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Discussion with Reviewers

R.P. Holmes: Is there any evidence that crystals retained beneath the epithelium in collecting ducts or papillae can "ulcerate" or otherwise move to the lumen surface or are you putting this forward as a hypothesis?
Author: From the evidence presented, I am concluding that crystals are ulcerating out of the tubules onto the papillary surface.

R.P. Holmes: You have mentioned three possible ways for crystals retained by the kidney to act as nidus for stone formation: (1) ductal occlusion by crystals, (2) attachment to "injured" epithelium, and (3) "ulceration" of subepithelial crystals. Do you believe that any one mechanism predominates or that all three may occur in stone formation?
Author: All of these processes may be operating in stone formation. Whether they occur separately or synergistically remains to be tested. Two important events appear to be crystals retention within the kidneys followed by their relocation to the papillary surface. Crystals may be retained either by forming large aggregates which occlude the ducts or by attaching to "injured" epithelial cells and then aggregating with other crystals. Crystal aggregates then ulcerate to the epithelial surface.

R.P. Holmes: Are you proposing that the ducts of Bellini are important sites for stone formation in humans following outgrowth from crystal agglomerates trapped within the ducts?
Author: Renal papilla and ducts of Bellini are generally regarded as a major site for stone formation in humans. The rat model has simply shown that chronic low grade hyperoxaluria can induce stones at the renal papilla indicating that chronic low grade hyperoxaluria may also be the cause of similar stone formation in humans.

A. Hesse: Most of the crystals which are taken up into the renal cells or into the interstitium will disappear with time. Do you have any idea what mechanism is responsible for this phenomenon? How do they disappear?
Author: Epithelial cells with the crystals they endocytosed may be dislodged and released into the urine. Crystals in the interstitium probably invoke an inflammatory response and are dealt with by macrophages. We have seen macrophages associated with interstitial calcium oxalate crystals. A study by Anderson and McDonald (1946) also showed macrophages in association with calcific deposits in papillary interstitium of human kidneys.

A. Hesse: If crystal endocytosis is a defensive mechanism in the prevention of stone formation, how is it controlled? Is it a defect in stone forming patients or only overtaxed.
Author: Recent studies of Lieske *et al.* (1994) have shown that crystal endocytosis by renal epithelial cells is mediated by many anions, including glycosaminoglycans, nephrocalcin and citrate. Perhaps, stone patients have a defect in production of such molecules.

Y. Nakagawa: How do rats form kidney stones in this model once becoming hyperoxaluric? Do rats consume inhibitors over the rate of production of inhibitors? Do the conditions creating hyperoxaluria cause production of abnormal inhibitors?
Author: Both oxalate and calcium oxalate crystals are

injurious to the epithelial cells (Hackett *et al.*, 1994). Inhibition of calcium oxalate monohydrate seed crystals is decreased in renal injury. Renal injury also results in acceleration of calcium oxalate monohydrate nucleation (Finlayson *et al.*, 1989). Thus, in hyperoxaluria-inducing rat models of nephrolithiasis, kidney stones are a consequence of higher supersaturation, lower crystallization inhibition and accelerated crystal nucleation. Whether crystallization inhibitors are defective or not available in sufficient quantities remains to be established.

R.L. Ryall: Numerous macromolecules and low molecular weight inhibitors have been shown to inhibit calcium oxalate crystallization in synthetic crystallization systems. Yet, to date, no single putative inhibitor has been unequivocally demonstrated to have an **undisputed** physiological **cause and effect** association with the formation of calcium oxalate stones. Do you think the inhibitor theory has been satisfactorily proven? Or is it still a truism awaiting scientific experimental verification?

Author: I agree. Inhibitor theory is a theory. Still, the major difference between idiopathic calcium oxalate stone formers and non-stone formers is reduced inhibitory activity in the urine of stone forming patients (Robertson, 1976; Pak and Galasy, 1980; Burns and Finlayson, 1983). Nature of substances and molecules responsible for the low inhibitory activity is, however, still not clear. I do not think a single inhibitor will ever be found responsible for this phenomenon in all the calcium oxalate stone formers. Crystallization involves a series of events which may be controlled by a variety of macromolecules. "Numerous macromolecules and low molecular weight inhibitors" which you say as having been shown to inhibit crystallization, may be involved in modulating different facets of the process of crystallization and may also be indicative of body's tendency for redundancy. Most of us are investigating only one or two aspects of crystallization, focussing on only one or two inhibitors and not the whole process. For example, we do not take into account interactions between various urinary macromolecules present in the urine. Here, I am reminded of an old parable about six blind men who want to know what an elephant is like. They touch different parts of his body and make their conclusions. To one who feels his sturdy body, elephant is like a wall. To the other, who gets hold of his tusk, elephant is like a spear. The third, who chances upon elephant's ears, compares him to a fan. The fourth likens him to a tree trunk by grabbing hold of his massive legs. The fifth, who holds up to the elephant's tail, compares him to a rope and the sixth, who touches the trunk, thinks of elephant as a snake.

In addition, as you pointed out, most of the work on crystallization inhibition has been carried out *in vitro* in synthetic inorganic systems where inhibition is actually defined by the physical test used to observe it. Some of the data may not necessarily be applicable to *in vivo* conditions in the urine. That is why it is important to study these phenomena in animal models.

As I indicated in the paper, promotary factors may also play crucial role in some stone forming patients. I consider higher supersaturation, renal injury and structural abnormalities of the kidneys as some of the factors that can promote the formation of kidney stones.

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