



Published in final edited form as:

Arch Esp Urol. 2017 January ; 70(1): 12–27.

HOW DO STONES FORM? IS UNIFICATION OF THEORIES ON STONE FORMATION POSSIBLE?

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Summary

There are two basic pathways for formation of calcium based kidney stones. Most idiopathic calcium oxalate (CaOx) stones are formed in association with sub-epithelial plaques of calcium phosphate (CaP), known as Randall's plaques, on renal papillary surfaces. Crystal formation and retention within the terminal collecting ducts, the ducts of Bellini, leading to the formation of Randall's plugs, is the other pathway. Both pathways require supersaturation leading to crystallization, regulated by various crystallization modulators produced in response to changing urinary conditions.

High supersaturation, as a result of a variety of genetic and environmental factors, leads to crystallization in the terminal collecting ducts, eventually plugging their openings into the renal pelvis. Stasis behind the plugs may lead to the formation of attached or unattached stones in the tubular lumen. Deposition of crystals on the plug surface facing the pelvic or tubular urine may result in stone formation on the Randall's plugs.

Kidneys of idiopathic stone formers may be subjected to oxidative stress as a result of increased urinary excretion of calcium/oxalate/phosphate and/or decrease in the production of functional crystallization inhibitors or in relation to co-morbidities such as hypertension, atherosclerosis, or acute kidney injury. We have proposed that production of reactive oxygen species (ROS) causes dedifferentiation of epithelial/endothelial cells into osteoblast type cells and deposition of CaP in the basement membrane of renal tubules or vessels. Growth, aggregation and melding of CaP crystals leads to the formation of plaque which grows by further calcification of interstitial collagen and membranous vesicles. Plaque becomes exposed to pelvic urine once the covering papillary epithelium is breached. Surface layers of CaP are replaced by CaOx through direct transformation or demineralization of CaP and mineralization of CaOx. Alternatively, or in addition, CaOx crystals nucleate directly on the plaque surface. Stone growth may also depend upon supersaturation in the pelvic urine, triggering further nucleation, growth and aggregation.

Keywords

Calcium Oxalate; Nephrolithiasis; Randall's Plugs; Randall's Plaques; Kidney Stones

INTRODUCTION

The first urinary stones were found in Egyptian mummies from 4000–5000 BC. Treatments for stones are mentioned in ancient Egyptian medical writings from 1500 B.C. Between the 5th and the 3rd century B.C., physicians swore to refrain from participating in stone surgery, as the Hippocratic Oath states “I will not use the knife, not even on sufferers from stone, but will withdraw in favor of such men as are engaged in this work.” In medieval times, surgery for stones, was performed by barbers who were called lithotomists. For 500 years, lithotomists travelled around Europe with their “lithotomy tables” cutting for stones. In 1561 the first suprapubic lithotomy was performed by Pierre Franco, to remove a bladder stone.

The National Health and Nutrition Examination Surveys (NHANES) which data has been helpful in the analysis of trends in kidney stone prevalence in the United States, demonstrated an increase in prevalence from 5.2% between 1988–1994 (1) to 8.8% between 2007–2010 (2). The Urologic Diseases of America project, funded by the NIDDK, estimated an annual cost of urinary stone disease to be \$10 billion, in 2012, making this the most costly urologic condition (Figure 1).

Aims to comply with the Hippocratic Oath have driven modern day urologists to find minimally invasive ways to carry out such surgeries. In 1929, Young was the first to report the performance of ureteroscopy. Since then, techniques have been developed, to avoid open surgical procedures and need to “cut for stone”. An important goal for urologists and scientists is to elucidate the etiology of stone formation in an effort to prevent stone episodes and recurrence.

Kidney stones are found either free in the urine in the renal collecting system, terminal collecting ducts, or attached to the renal papillary surface or epithelial surface of the terminal collecting ducts (3–5). In 1937, Randall proposed that kidney stone formation requires “an initiating lesion that precedes the formation of a renal calculus” (6). He reported that 17% of the 429 pairs of kidneys examined at autopsy showed “deposition of calcium in the walls and intertubular spaces of the renal papilla.” He described these plaques as a consequence of “a natural reparative process to some form of tubular damage”. After dissection of 1154 pairs of kidneys, Randall found that 19.6% had evidence of a calcified lesion in at least one renal papilla and 65 kidneys had a primary renal stone attached to the papillary plaque (7, 8). He suggested that interstitial deposits of calcium phosphate (CaP) and calcium carbonate erupt on the renal papillary surface forming pre-calculus lesion type I (8), currently known as Randall’s plaques (9). He also suggested that excessive urinary supersaturation or “hypersecretion” results in crystallization of stone forming salts and plugging of the collecting duct, forming pre-calculus lesion type II (8), now called Randall’s plugs (10). Thus Randall has suggested two pathways for the formation of kidneys stones. One through changes in the renal parenchyma and formation of Randall ‘s plaques (RPL) and another requiring alteration in the urinary composition or supersaturation and development of Randall’s plugs (RPG).

Randall's Plaque

Morphological Characteristics of Randall's Plaques—Anatomical evidence for RPL has been demonstrated by various investigators including Stoller (9), Haggitt and Pitcock (11), Cooke (12,13) Evan (9,14) and Khan (4,10,15,16). Even though renal papillae of both the stone formers and non-stone formers have plaques, they are more common in kidneys from stone-forming patients (7). Haggitt and Pitcock (17), found alizarin red positive laminated spherules in the renal interstitium which on closer examination by TEM were found associated with collagen fibers not only in the interstitium, but also in the basement membrane of the collecting ducts. Interestingly, all RPLs do not support stone formation, as kidneys of non-stone formers also show the presence of calcified deposits in the renal interstitium (17). Cooke and associates (12,13), examined 62 normal kidneys. Four of them had interstitial calcifications located mostly in the basement membrane of the loops of Henle extending into the medullary interstitium as well as the surrounding collecting ducts and blood vessels. Stoller et al performed high resolution radiography of cadaveric kidneys (9) and found that 57% of the kidneys had sub-epithelial RPL which extended deep within the papillae associated with collecting ducts and vasa recta. Evan and associates analyzed renal papillae from a variety of stone patients (18). They concluded that idiopathic calcium stones develop attached to the sub-epithelial RPL (19), without any signs of cell injury, inflammation, interstitial fibrosis, and intratubular crystal deposition. They hypothesized that interstitial crystal deposits of idiopathic stone formers migrate from the basement membrane of the loops of Henle into the surrounding interstitium and become associated with type 1 collagen, fusing into a “syncytium in which islands of mineral appear to float in an organic sea” (18,20). In addition to CaP crystals, plaques also contain organic matrix positive for osteopontin (OPN) (21,22), heavy chain of inter- α -inhibitor (23,24) collagen (13,17,25) and zinc (26).

Khan et al. performed detailed scanning (SEM) and transmission electron microscopic (TEM) analysis of the RPL and renal papillary tissue at the stone attachment site (16). The plaque appeared as a small protuberance (Figure 2A). SEM analyses showed plaques to be aggregates of spherical CaP crystals mixed with fibers of varying thickness and other cellular products (Figure 2B).

There were two distinct types of interstitial CaP deposits which in addition to crystals also contained membrane bound vesicles, collagen and some unidentified fibrillar material (16). Needle shaped electron dense entities, most probably nucleating CaP crystals, were found in some of the vesicles (Figure 2C). Basement membrane of the renal tubules as well as surrounding interstitium was inhabited by 0.5 μm to 2 μm in diameter concentrically laminated spherical deposits (Figure 2D). The center of the calcification contained amorphous to needle or thin plate shaped crystals (Figure 2E). Other types of calcification, generally located in the interstitium and under the papillary epithelium, included dark and dense deposits of elongated strands mixed with aggregating spherulitic crystals. Some of the collagen fibers, particularly on the periphery, appeared calcified (Figure 2F). Energy dispersive x-ray microanalyses of the spherical crystals showed them to contain calcium and phosphorus, suggestive of their CaP nature. Electron diffraction analyses identified the

crystals as hydroxyapatite and also revealed that the center of the calcification was more crystalline than the periphery (16).

Pathogenesis of Randall's Plaques: Hypoxia, Reactive Oxygen Species and cell Injury—Randall's plaque formation and its transformation into a stone nidus occurs in at least four distinct phases. First, there is the initial crystal deposition in the papillary interstitium in and around renal tubules and blood vessels, which is followed by the 2nd phase-growth and expansion. In the 3rd phase, epithelium covering the plaque is breached, exposing it to the pelvic urine. The 4th phase involves deposition of CaOx on the plaque, in which the outer layer of hydroxyapatite is replaced by CaOx or CaOx nucleated on the crystal associated organic matrix.

Most of the understanding of pathogenesis is based on the urinary characteristics of stone formers and morphological studies of human renal tissues. Even though results of such observational studies can only provide a snapshot at the end of a long process, they are useful in development of theories about stone pathogenesis and interpreting results of experimental studies performed to determine the mechanistic details. Randall's plaque formation is associated with low urinary volume, low urinary pH and high urinary excretion of calcium (18,20,27). Calcium phosphate crystals either form directly in the basement membrane of the loops of Henle (28,29) or initially form in the supersaturated urine of the renal tubules and then transported into the basement membrane or interstitium (30).

Randall proposed that plaque formation involved damage to the epithelial lining of the collecting ducts and interstitial connective tissue. Calcium deposition appeared to primarily occur in the basement membrane of the damaged epithelium forming ring like structures around the tubules. He suggested that interstitial subepithelial deposits of calcium phosphate and calcium carbonate, arising from pathologic conditions of the renal papilla, erode through to the papillary surface forming the plaque (8). Vermooten also suggested that deposition of calcium in the papillary interstitium and basement membrane of the collecting ducts was associated with senescence (31).

Evan et al. however found no signs of cell injury, inflammation, or interstitial fibrosis (19,25,32). Khan et al however, showed calcification in association with damaged renal tubules and suggested that crystal deposition started within the membranous vesicles formed as a result of cellular injury (16). They further proposed that injury and inflammation play a significant role in the formation of RPL (33–35). Recent studies (36) of the plaque by Taguchi et al demonstrated upregulation (>2-fold) of LCN2 (lipocalin 2), IL11 (interleukin 11), PTGS1 (prostaglandin-endoperoxide synthase 1), GPX3 (glutathione peroxidase 3), and MMD (monocyte to macrophage differentiation associated) and downregulation (0.5-fold) of SLC12A1 (solute carrier family 12 member 1) and NALCN (sodium leak channel, non-selective) genes in the plaque tissue. These genes are associated with activated mitogen-activated protein kinase, the Akt/phosphatidylinositol 3-kinase pathway, and proinflammatory cytokines involved in the development of oxidative stress and causing renal injury.

The scenario presented above is based upon renal tubular epithelium playing a critical role in the development of Randall's plaques. Recent epidemiological studies, however, suggest that renal vascular system may also play a significant role in plaque development (33,37–39). Stoller et al showed that plaque is not limited to the basement membrane of the loops of Henle but reaches deep into the interstitium and basement membrane of the vasa recta (37). Thus plaque formation is somewhat similar to calcified plaques in the renal arteries and capillaries. There is evidence for the association between stone formation and aging and cardiovascular diseases, including hypertension, myocardial infarction, diabetes, chronic kidney disease, metabolic syndrome (38–40).

Idiopathic CaOx Stone formation is a risk factor for development of hypertension. Uric acid stone formers have higher prevalence of diabetes mellitus. Hypertensive and diabetic patients are at a greater risk for the formation of kidney stones. In addition, tissue hypoxia, production of reactive oxygen species and development of oxidative stress are characteristic features of vascular diseases with renal association (34,35,39). Results of a number of clinical and experimental investigations suggest that reactive oxygen species (ROS) may be involved in the pathogenesis of idiopathic stone disease (35,41,42). CaOx stone patients excrete significantly higher amounts of N-acetyl- β -glucosaminidase (NAG), β -galactosidase, α -glutathione S-transferase (α -GST), malondialdehyde (MDA) and thiobarbituric acid-reactive substances (TBARS) (41), which are biomarkers of injury and production of ROS. Urinary 8-hydroxydeoxyguanosine(8-OHdG), a marker of oxidative damage of DNA, which is increased in stone patients and positively correlated with tubular damage as assessed by urinary excretion of NAG (43). Antioxidant deficit is common in recurrent idiopathic stone formers and was unrelated to the presence or absence of stones (44). It has been suggested that “renal disorders which lead to OS appear to be a continuum. Stress produced by one disorder may trigger the other under the right circumstances” (39).

The association between aging and kidney stone risk and prevalence was observed by Scales et al, 2012 (Figure 3) (2). Prevalence, history of kidney stones and history of passing stones was more frequent at the sixth decade of age (95% CI). In addition, a multivariate regression model predicting history of kidney stones demonstrated an odds ratio of 2.18 for age over 60 y/o (95% CI). A possible proposed mechanism may be through glycation end products (AGEs).

AGE's, are the product of non-enzymatic glycation and oxidation of nucleic acids, proteins and lipids. This process has been shown to be associated with aging and pathologic conditions such as hyperglycemia among others (45) (Figure 4).

Recent findings demonstrate that accumulation of AGE products might be involved in vascular calcifications associated with diabetes (46). Diabetic cellular mechanisms of disease include glucose-induced excessive formation of reactive oxygen species, increased glucose flux through the polyol pathway, formation of advanced glycation end-products and oxidative stress (47), which result in decreased renal oxygenation and subsequent activation of hypoxia-inducible factors. Ultimately this chain of events leads to EMT. These pathways have been demonstrated as a chain reaction involving HIF1, TGF-B and increased

expression of AQP1 channels (48). This hypoxic pathway has been demonstrated to lead to chronic kidney disease (49, 50).

The relationship between diabetes and vascular calcification are thought to involve reactive oxygen species which induce vascular smooth muscle cell (VSMC) transformation to osteogenic phenotype by regulating RUNX-2 transcription factor (51,52). Advanced glycation end-products in diabetic patients and older individuals can promote vascular calcification mediated by NADPH oxidase induced reactive oxygen species (53). Cytokines such as IL (interleukin)-1 β , IL-6, IL-8, (TNF) tumor necrosis factor- α , TGF (transforming growth factor)- β produced by macrophages also induce transformation of VSMCs (54). Inflammatory cells also produce proteolytic enzymes such as metalloproteinases (MMP)-2 and -9 which degrade matrix and promote calcification (55–58). In the diabetic state, hypoxia mediated induction of profibrotic and proinflammatory molecules, such as TGF- β and TNF- α , induce proteinuria, tubulointerstitial fibrosis, infiltration of immune cells and EMT progressing to chronic kidney disease (48).

Renal cells which undergo EMT and specifically undergo osteoblastic differentiation, start the process of tissue calcification via specific cellular functional changes, such as loss of epithelial features and gaining osteogenic features similar to the changes seen in VSMC (59). Explanations of this specific phenomenon have included high extracellular calcium as a pivotal element in EMT to osteoblastic differentiation. This osteoblastic differentiation is believed to be the common precursor of vascular and kidney interstitial calcifications (Figure 5) (48). In addition, association of diabetes with higher incidence of uric acid stones is due to impaired kidney ammoniogenesis, alkalinization defects, and abnormal low pH in urine (60).

In the hypertensive state, superoxide anion (O₂⁻) levels are increased 10 fold, leading to IMI supersaturation by significantly enhanced NaCl reabsorption in the ATLH in animal models. In addition, increased levels of superoxide lead to increased production of hydroxyl radicals, which in turn leads to oxidative damage to DNA, proteins, and lipids (61). In a similar study in hypertensive rat kidneys, it was demonstrated that precapillary oxygen shunting reduced the pO₂ of cortical nephrons. The pO₂ was reduced further in spontaneously hypertensive rats because of less efficient oxygen usage for Na⁺ transport (62). HTN induced hypoxia is associated with solute supersaturation via the pathways described above. RAS have been implicated as the hypoxic mediated renal insult in hypertension. Explanation of RAS involvement is related to the increased angiotensin receptor, oxidative stress, and precapillary oxygen shunting leading to renal hypoxia. The high renal oxygen demand is due to tubular oxygen consumption necessary for solute reabsorption. The combination of hypertension, angiotensin II, and oxidative stress initiates events leading to renal damage and reduced function. There is a greater than a 10 fold increase in osmolality and decrease in oxygen-carrying capacity between renal cortex and papillary tip, which is believed to be the consequence of the laminar microvascular blood flow changes to turbulent flow at the tip of the inner papilla (51). Tissue hypoxia is now recognized as a unifying pathway to chronic kidney disease.

Obesity has been linked to intracellular hypoxia and production of reactive oxygen species via fat metabolism. It has been demonstrated that hypoxia is involved with dysregulated production of adipocytokines. Adipocytokines have been identified to be partly responsible for obesity-linked metabolic disorders. Studies demonstrate that adiponectin mRNA is downregulated during hypoxia (63). Adiponectin is an adipocytokine. It is associated with prevention of inflammation, atherosclerosis, and improved lipid and glucose metabolism. The connection between vascular inflammation and atherosclerotic plaque formation and adiponectin is via increased expression of tissue inhibitors of metalloproteinases (TIMPs) in human macrophages through IL-10 induction. Adiponectin then prevents tissue degradation in vasculature by matrix metalloproteinases (MMP's) thus preventing rupture of atherosclerotic plaque and possible acute thrombotic episodes (64). It may be that in nephrolithiasis, inner medullary interstitial hypoxia, in the setting of obesity, may contribute to tissue disruption and Randall's plaque ulceration via this mechanism (Figures 4, 5).

Deposition of CaP in the renal medullary interstitium i.e. the formation of Randall's plaques has also been reported in a variety of animal models (65). However, none have shown a CaOx stone growing on a subepithelial plaque, even after experimental induction of hyperoxaluria (66). Nonetheless, such models provide interesting information on the role of various elements involved in the initial stages of plaque formation. NHERF-1 (sodium-hydrogen exchanger regulatory factor-1) and THP (Tamm Horsfall Protein) null mice produce interstitial apatite plaques in the renal papillae, similar to that of human plaque (67). THP null mice also produce both intratubular and interstitial CaP deposits (Figure 6).

NHERF null mice are hypercalciuric and hyperphosphaturic and produce small interstitial CaP deposits associated with both the loops of Henle and inner collecting ducts. A few smaller deposits are also seen in the basement membrane of their loops of Henle. 88% of the 15 month old THP-null mice produce numerous large deposits in the renal papillary interstitium. The deposits consisting of HA, surround both the thin loops of Henle as well as inner medullary collecting ducts without involving their basement membrane. The mice are hypercalciuric and hypocitraturic. Their urine is supersaturated with respect to brushite (68). OPN null mice also produce interstitial CaP deposits in the renal papillae without an increase urinary excretion of calcium, phosphorus (69). Urine of the OPN null mice is also not supersaturated with respect to brushite. Interestingly both THP and OPN are inhibitors of mineralization (70) are anti-inflammatory and have been identified in the renal interstitium (22,71). Targeted ablation of ABCC6 gene, responsible for generalized arterial calcification and ENPP1 gene which encodes for ectonucleotide pyro-phosphatase/ phosphodiesterase 1 in mice leads to late onset ectopic mineralization mainly manifested in skin, eyes and cardiovascular tissues. When placed on an accelerated diet of high phosphorus and low magnesium these mice develop extensive mineralization in the renal interstitium mainly restricted to renal medullary tubules and arcuate and renal arteries (72). Magnesium is a well-known inhibitor of mineralization (73). Mineral deposits consisted of CaP and stained positive for both OPN and fetuin. The production of both OPN, THP, fetuin and other macromolecular mineralization inhibitors is regulated by reactive oxygen species (74).

Pathogenesis of plaque formation is considered mostly a physicochemical process (75). We (76) as well as some others (77) have proposed that Randall's plaque formation is similar to vascular calcification in the kidneys in which vascular smooth cells (VSMC) acquire osteogenic phenotype (78–80), when exposed to higher concentrations of calcium and phosphate (81–84). Expression of osteoblast specific genes is increased and smooth muscle cell specific genes are decreased (85,86).

Bone morphogenetic proteins, BMP 2 and BMP 4, and Wnt signaling pathways are activated through up-regulation of transcription factor, Runt-related transcription factor 2 (RUNX2). Reactive oxygen species are likely involved in the VSMC transformation to osteogenic phenotype by regulating RUNX-2 transcription factor (51,52). It is our hypothesis that oxidative stress caused by abnormal urinary environment such as hyperoxaluria, hypercalciuria and hypocitraturia and formation of crystals, produce specific changes in the renal epithelial cells (4).

Tubular cells lose their epithelial character and become osteogenic. We tested our hypothesis in a rat model of hyperoxaluria by performing genome wide analysis of differentially expressed genes in the kidneys and determined changes consistent with the dedifferentiation of epithelial cells into bone producing cells. Genes for runt related transcription factors (RUNX1 and 2), zinc finger protein Osterix, bone morphogenetic proteins (BMP2 and 7), bone morphogenetic protein receptor (BMP2R), collagen, osteocalcin, osteonectin, osteopontin (OPN), matrix-gla-protein (MGP), osteoprotegerin (OPG), cadherins, fibronectin (FN) and vimentin (VIM) were up regulated while those for alkaline phosphatase (ALP) and cytokeratins 10 and 18 were down regulated (87). In an animal model of CaP nephrolithiasis, the genetic hypercalciuric rat, basal levels of BMP2, RUNX2, and Osterix are increased in the kidneys with intratubular calcium phosphate deposits (88). Knockdown of the vitamin D receptor in the rats reduced the expression levels of BMP2, RUNX2, and Osterix as well as CaP crystal deposition in the kidneys.

Support for the transformation of renal epithelial cells into osteogenic phenotype comes from many cell culture studies. Exposure to CaOx crystals increased vimentin expression in normal rat kidney epithelial cells in (89). The inner renal medullary collecting duct epithelial cells of rats grown in calcifying media produced calcifying nodules positive for bone morphogenetic proteins (90). When MDCK cells were grown in monolayers on a collagen gel (91,92), small blisters/domes/nodules developed after 30 days. The nodules contained aggregated spherulites of needle shaped CaP crystals. MDCK cells grown in agar produced spherical colonies enclosing CaP crystals (92,93).

Thus plaque and associated idiopathic CaOx stone formation involves many steps (Figure 7). It starts mainly with the development of supersaturation due to hyperoxaluria, hypercalciuria and hypocitraturia. There may or may not be the formation of and retention of intratubular CaOx crystals. There is a transformation of epithelium/endothelium to osteogenic phenotype. CaP phosphate crystals are deposited on the basal side. Any CaP formed in the tubular lumen is transported into the basement membrane/interstitium through phagocytosis or through a loss of tubular epithelium. Interstitial CaP crystals aggregate, coalesce forming a calcification front. Collagen becomes involved and is calcified. The front keeps growing

eventually reaching the papillary epithelium which is breached through the actions of metalloproteinases. Once epithelium is eroded, CaP is exposed to the pelvic/calyceal urine. Pelvic urine is metastable with respect to CaOx, comparatively slow moving and at places stagnant, conditions promoting the nucleation of CaOx on the exposed plaque surface. By addition of more crystals a stone is formed attached to the Randall's plaque.

Randall's Plugs

Morphological Characteristics of Randall's Plugs—Randall's plugs are crystalline deposits clogging the terminal collecting ducts, ducts of Bellini and their openings into the renal pelvis (Figure 8). Cystine, brushite, CaP stones in primary hyperparathyroidism, and CaOx stones of primary hyperoxaluria and post-bariatric surgery have all been found attached to Randall's plugs (15,18,94).

A recent study (94) has provided newer evidence that even some idiopathic CaOx stones may develop on Randall's plugs formed as a result of intratubular deposits of CaP. It should however be noted that no one has yet reported an idiopathic stone growing over a Randall's plug. The plugs are generally exposed to two different urinary environments, one is bathed in the pelvic urine and another in tubular. As a result, the two sides of the plugs may have different crystalline composition.

We performed morphological examination using light and scanning and transmission electron microscopy of Randall's plugs, consisting of CaOx crystals, removed from the terminal collecting ducts of a patient with primary hyperoxaluria with bilateral renal calculi (15). Stone composition was determined by x-ray diffraction and energy dispersive x-ray microanalysis. X-rays revealed bilateral staghorn calculi. All stones consisted of CaOx mixed with small amount of apatite. A large stone measuring 1.7×1.5×0.8 cm, was found completely lodged inside the renal papilla. X-ray diffraction analyses showed that stone comprised of 29% CaOx monohydrate, 62% CaOx dihydrate, 4% hydroxyapatite and 5% organic material.

Papillary tissue showed dilated inner medullary collecting ducts and ducts of Bellini, interstitial fibrosis, focal tubular epithelial hyperplasia, interstitial calcification and intratubular deposits of birefringent CaOx crystals/small stones. Transmission electron microscopy showed an enlarged interstitium, with myofibroblasts, abundant collagen, fibrillar material as well as electron dense deposits of CaP. Cellular degradation products including membrane bound vesicles concentrically laminated spherulites with needle shaped apatite crystals were associated with the large interstitial CaP calcification. Basement membrane of the tubular epithelium showed thickening and layering. Some tubular epithelial cells appeared necrotic with swollen mitochondria and vesiculating endoplasmic reticulum. At places tubular epithelium lifted from the basement membrane while at other sites epithelial cells ruptured releasing their contents into the tubular lumen. Thus the kidneys of this patient with primary hyperoxaluria has Randall's plug of CaOx within the terminal collecting duct and Randall's plaque in the papillary interstitium.

Randall's plugs of the brushite stone formers which are mostly brushite, also cause serious damage to the surrounding papillary tissue. There are dilated tubules, with localized loss of

epithelium, interstitial fibrosis and scattered interstitial Cap deposition or plaque formation (95).

Pathogenesis of Randall's Plugs—Randall proposed that plugs or as he called them, Type II lesions, are formed as a result of excessive urinary supersaturation and necrosis of the tubular epithelium. The presence of plugs in patients who excrete highly supersaturated urine supports Randall's position. Cystinurics develop cystine stones over cystine plugs (96). Patients with primary hyperoxaluria develop CaOx stones in addition to CaOx plugs (15,18). In cases where urine is supersaturated with respect to more than one salt, plugs as well as stones contain more than one type of mineral (18). The plugs form, in part, as a result of higher supersaturation with respect to the precipitating salt (6,7,17) and are generally associated with renal tubular injury and focal inflammation as indicated by Randall (7).

Use of excessive amounts of medications such as magnesium trisilicate, ciprofloxacin, sulfa medications, triamterene, indinavir etc. can lead to the formation of kidney stones (97), most likely through plugging of the terminal collecting ducts. Poorly soluble dietary components can also crystallize in the renal tubules and produce kidney stones. Melamine in the baby formulas led to the formation of kidney stones in thousands of infants (98).

Results of animal model studies also support the contention that supersaturation is the driving force for the formation of stones associated with plug formation. Experimental induction of mild hyperoxaluria with increased urinary CaOx supersaturation leads to the deposition of CaOx crystals in the collecting ducts of rat kidneys (Figure 9) (99). Similarly urolithiasis induced by the administration of oxamide in diet leads to urinary excretion of oxamide crystals and their deposition in the terminal collecting ducts of the rat kidneys (100–102).

Plugging involves not only crystal formation and growth, which can be explained through urinary supersaturation, but also crystal retention within a tubule with flowing urine. It should therefore depend not only on supersaturation but also tubular dimension, crystal growth rates, diurnal variations in urinary flow, as well crystal aggregation. The inner diameter of the inner medullary collecting ducts ranges between 35–60 μ m and of the ducts of Bellini 60 to 100 μ m (3). Single crystals are too small to be able to occlude the tubules (5) and may require attachment to the tubular wall (103,104) followed by growth and/or aggregation with other crystals (3). Alternatively, crystals formed in the glomerular filtrate can become large enough to be trapped in the collecting ducts, particularly those at the end of the long loops of Henle. Aggregation can increase the size in a short span of time (105) and stone formers have been reported to excrete large crystal aggregates (106).

It has been suggested that in the collecting ducts urine flows in discreet boluses propelled by the peristaltic waves (107). Such a movement would result in significant turbulence increasing the possibility of crystal contact and aggregation (108). Plug formation may also be assisted by the fact that luminal diameter the ducts is much larger than the opening of the ducts of Bellini, which are narrow and slit-like (104). Urine may flow at different velocities through the tubule (109), slower near the at the epithelial surface. As a result, crystals would travel at different speeds, faster in the middle of the urinary stream and relatively slower at

the tubular epithelium. The crystal moving near the epithelium, would not only have a chance to grow bigger compared to the fast moving ones but also have increased contact time with the epithelial cells. Renal tubules also drain upward, which would affect the crystal movement. Taking into account these hydrostatic factors, Robertson concluded that crystals forming at the end of the descending limb of the loops of Henle and travelling close to the tubular epithelium can become large enough to be trapped in the tubular lumen (110).

Ductal plugging is often associated with small concretions in the inner medullary collecting ducts as well as renal pelvis and calyx (15,27). Such stones have been seen in patient with hyperoxaluria (15) as well as patients with uric acid and cystine stones (27). Urine behind the ductal plugs should be stagnant and may promote crystal formation and aggregation because of high supersaturation which normally exists in these patients. Similarly, pelvic urine around the plugged papillae may also be stagnant and promote stone formation through crystallization in a supersaturated urine.

Thus plugs as well as other free stones associated with plugs may form freely in the urine through crystal formation, growth and aggregation. Animal model and tissue culture studies have shown that crystals are injurious to the epithelial cells. Injured cells are prone to support crystal attachment. Once crystals are retained, ensuing injury would accelerate further crystallization and retention leading to more stone formation.

CONCLUSIONS

A Unified Theory on Pathogenesis of Kidney Stones

Stone formation is a complex multistep process. A variety of theories have been presented to explain stone pathogenesis based upon urinary supersaturation, crystallization inhibitors, and vascular insult. Kidney stones being mostly crystalline, all of the theories agree that stone formation requires urinary supersaturation with respect to the crystalline component (4,30,111–116). Since crystallization may lead to tubular blockage and injury, affecting renal function, kidneys normally produce mineralization inhibitors (70,74,117,118). Degree of supersaturation, which is dependent on increased urinary excretion of ions involved in crystallization (116, 119–123) would however, determine whether stones start in the tubular lumen or the renal interstitium. As discussed above a variety of stones start as a result of high supersaturation and crystallization in the tubular lumens. But the most common kidney stones, the idiopathic calcium oxalate stones, are formed attached to subepithelial plaques of calcium phosphate. Mechanism involved in the formation of plaques are however uncertain. It is proposed that plaques start in the basement membrane of the loops of Henle (27) mostly because of high urinary calcium (124). But there is no experimental proof. High urinary calcium in rodents does not lead to the formation of interstitial plaques (125–127).

Randall's plaques are common even in non-stone forming individuals (113,128) as well as in stone patients with plug based kidney stones (15). Perhaps plaque formation, at least in some stone patients, is a result of papillary injury caused by plugging and secondary to the tubular blockage. Another theory proposes that plaques are formed secondary to vascular insult near tip of the renal papilla (37). The hypothesis is supported by the fact that majority of stones are associated with chronic kidney diseases such as hypertension, atherosclerosis,

hyperlipidemia etc. We have previously shown that production of reactive oxygen species and development of oxidative stress are common in both the idiopathic stones formers and chronic kidney disease patients (34, 39). It is remarkable that patients with hypertension can eventually develop idiopathic kidney stones and kidney stones may lead to hypertension. We have proposed a hypothesis combining the current theories (4,10).

Renal epithelial cells of stone formers become stressed when challenged by increased urinary excretion of calcium/oxalate/phosphate and/or decrease in the production of functional crystallization inhibitors or perhaps with renal insults, and the abnormal process of aging. There is production of reactive oxygen species causing de-differentiation of renal epithelial/endothelial cells into osteoblast like cells. CaP crystals are deposited in the renal tubular epithelial/vascular endothelial basement membrane. Small spherical units of CaP grow from less than a micron to a few microns in diameter and spread out beyond the basement membrane. CaP spherulites aggregate and form larger deposits or plaques which increase in size by further addition of crystals on the periphery. The growing plaque comes into contact with collagen fibers and membranous degradation products which also undergo calcification (16).

Interstitial CaP deposition produces localized inflammation and fibrosis, providing substrate for further calcification until the front reaches the papillary surface. Urothelium is most likely breached through the activity of matrix metalloproteinases, loss of cellular polarity, architecture, and cell to cell junctions. Once the surface epithelium loses its integrity, the plaque is exposed to the metastable pelvic urine, and becomes a nidus for stone formation. Macromolecules present in the pelvic urine and are produced by renal epithelial cells in response to reactive oxygen species (74), deposit on the plaque surface. Surface layers of HA are replaced by CaOx through direct transformation or demineralization of CaP and mineralization of CaOx. Alternatively, or in addition, CaOx crystals directly nucleate on the macromolecules deposited on plaque surface. Further growth is again dependent upon the urinary supersaturation and is accomplished by crystal nucleation, growth and aggregation on the periphery.

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(*of special interest, **of outstanding interest)

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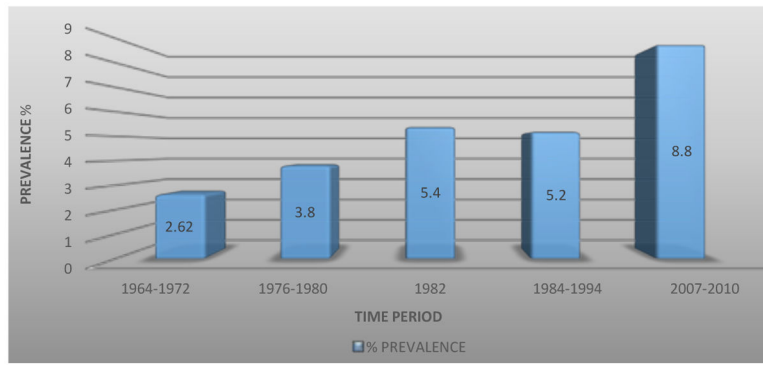


Figure 1.

The prevalence of kidney stones has been steadily increasing in the last five decades.

According to the last NHANES data analysis the unadjusted overall prevalence of kidney stones has increased to 8.8%, with 10.6% and 7.1% in men and women respectively (C. Scales et al, 2012).

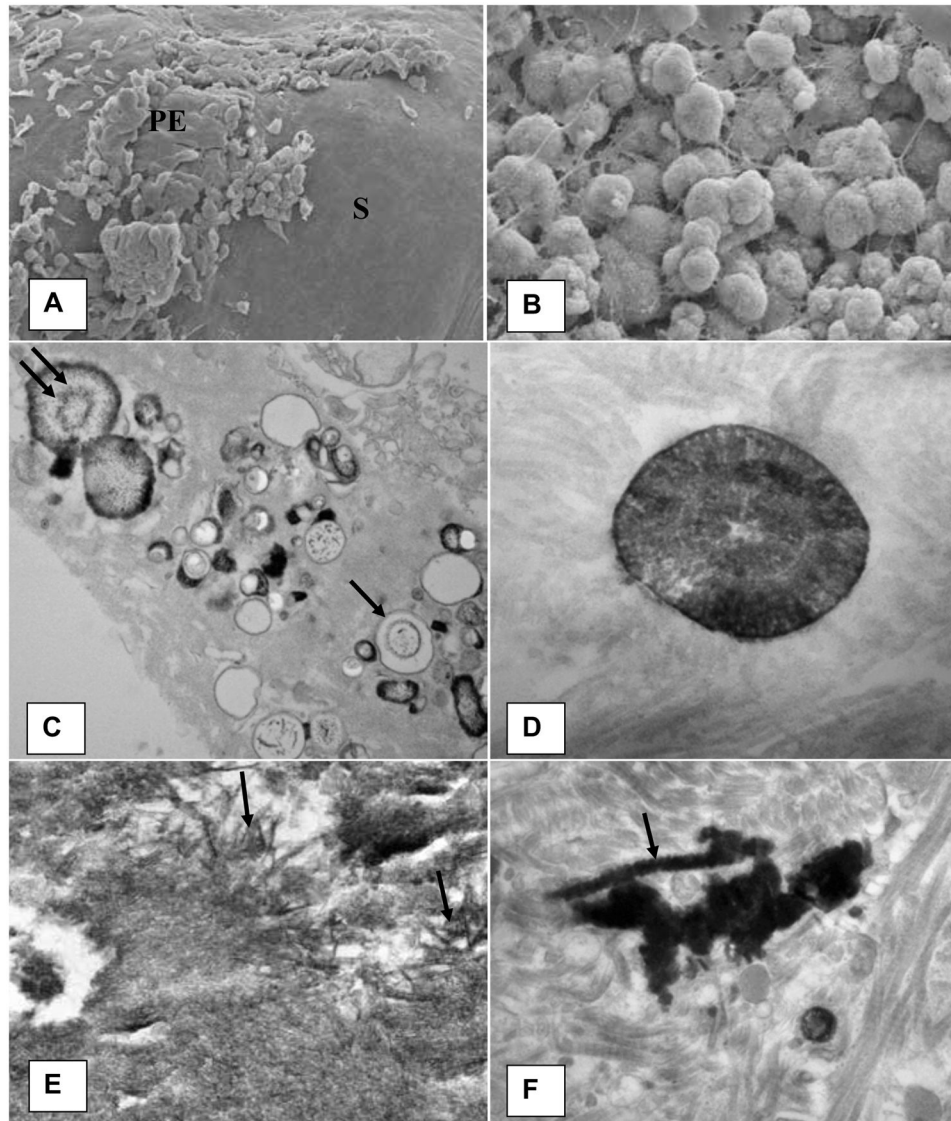


Figure 2. Microscopic appearance of the Randall's plaque. A). Plaque appears as a bulge on the papillary surface. Epithelium (PE) is eroding exposing sub-epithelial amorphous layer (S). B). Spherical deposits of CaP mixed with fibrous material are present under the papillary surface epithelium. C). Calcification consisting of large and small vesicles (arrows), some with crystalline material inside (double arrow) are present embedded in an amorphous to granular to fibrous substrate. D). Concentrically laminated spherical unit surrounded by fibrous substrate. E). Calcification consisting of large dark deposits. Center of the deposit showing plate-like individual crystals (arrows). F). Calcification surrounded by what appear to be collagen fibers with distinct banding pattern (arrow). Arrow points to a calcified collagen fiber showing beaded exterior. Adapted from Khan and Canales, 2015, *Urolithiasis* 43 Supplement 1:109–123 (10).

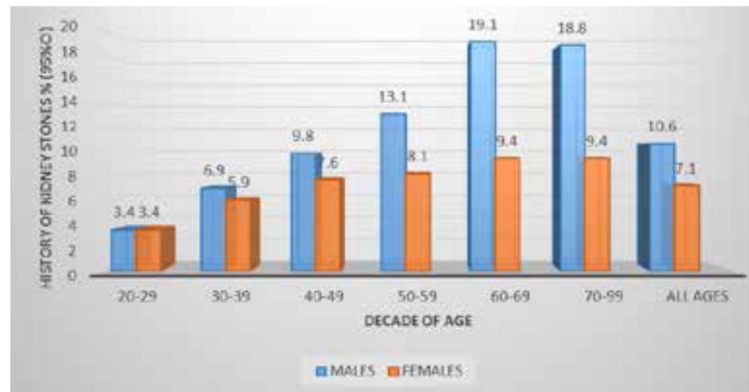


Figure 3.

The history of having kidney stones increases with age according to NHANES data analysis on unadjusted percent prevalence of kidney stones by age and gender. These findings were more pronounced in males compared to females, 19.1% vs 9.4% respectively, in their sixth decade of age. (C. Scales et al. 2012)

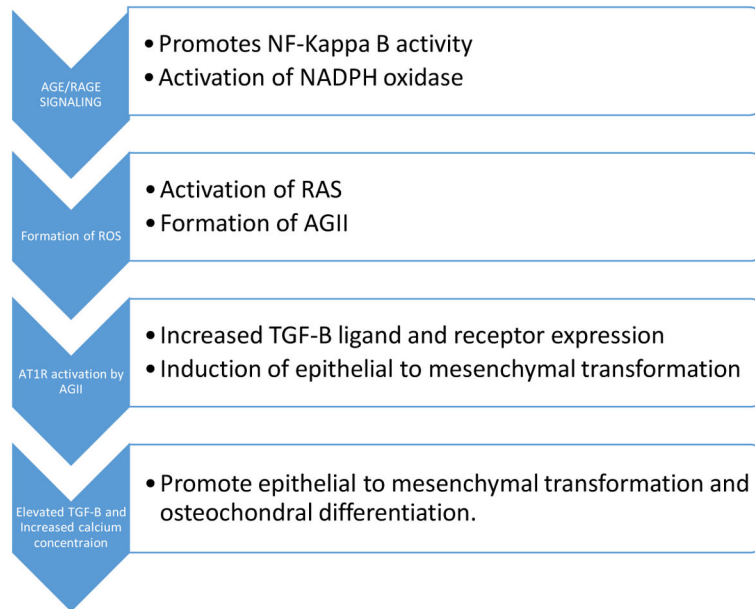


Figure 4. AGE/RAGE signaling pathway for the formation of NADPH oxidase mediated reactive oxygen species (ROS), angiotensin receptor activation and induction of TGF-B expression. TGF-B have been proposed to be a major regulator of the induction of epithelial to mesenchymal transformation (EMT) in the kidney (45). TGF-B and increased calcium concentration promote mesenchymal to osteochondral cell differentiation (59).

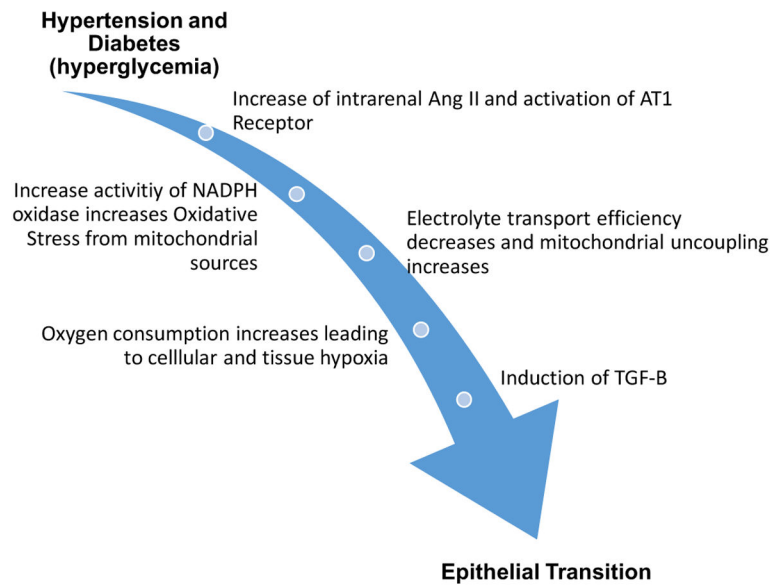


Figure 5. Relationship between Diabetes (hyperglycemia) and Hypertension with Hypoxia. Hypertension mediated increased angiotensin receptor activity and oxidative stress and diabetic mediated mitochondrial dysfunction from excess tubular oxygen consumption lead to a common pathway of tissue hypoxia. The results will include induction of TGF-B among other factors. TGF-B among other factors. TGF-B will be responsible of epithelial to mesenchymal transformation (48).

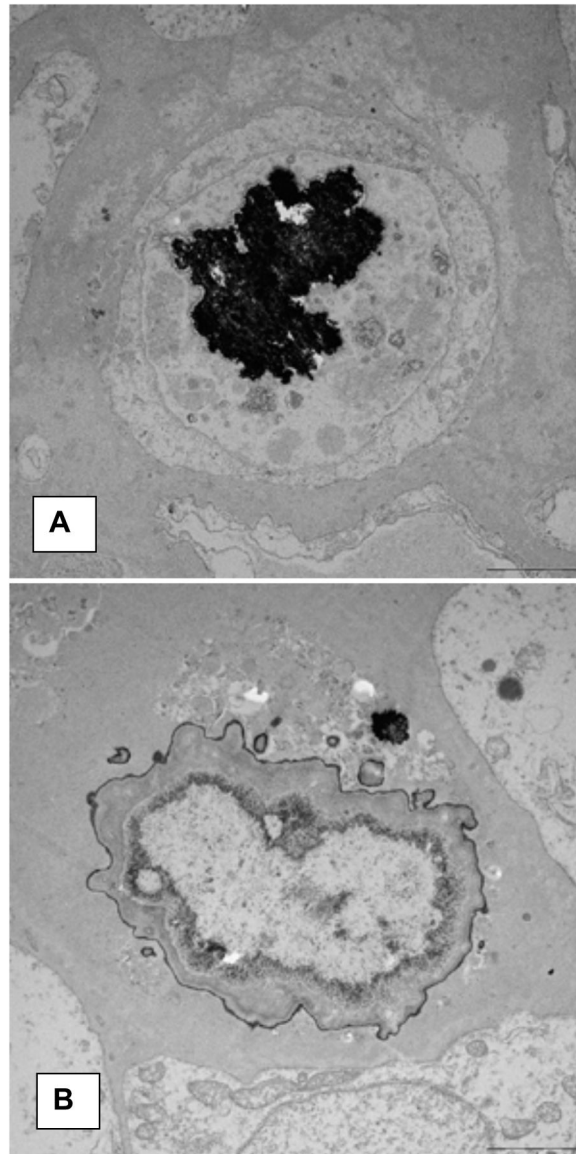


Figure 6. Transmission electron microscopic analyses of the renal papilla of THP null mice. A). Calcified deposit inside a loop of Henle. B). Calcification in the renal interstitium. Copious amount of organic material is mixed with dark staining CaP crystals.

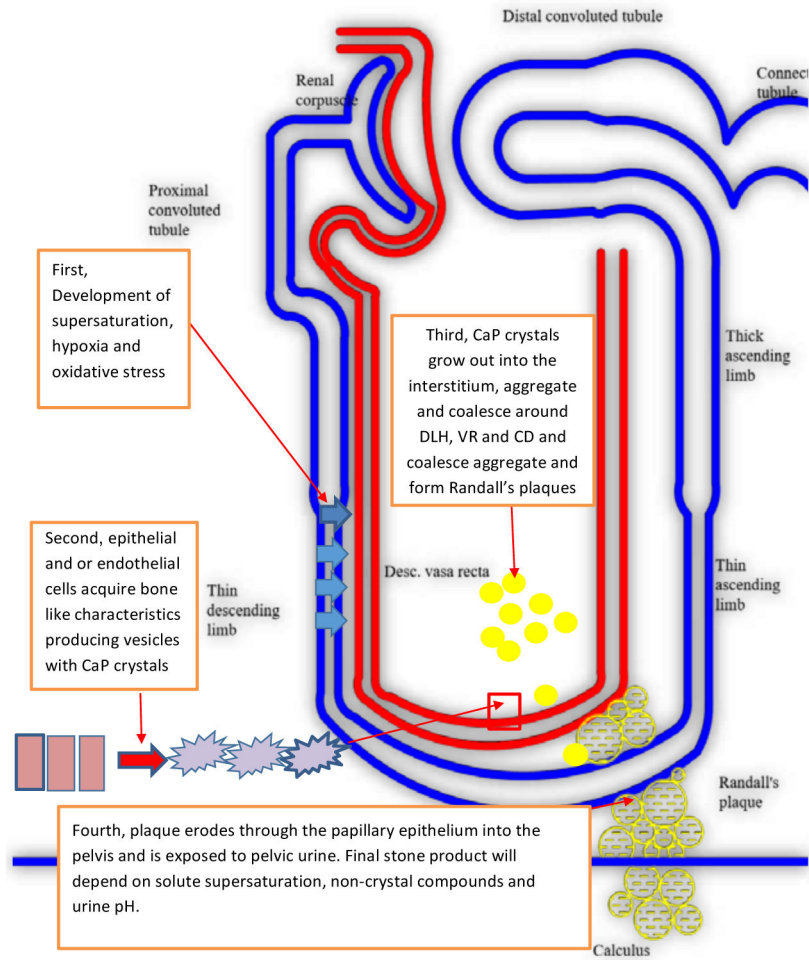


Figure 7. Schematic presentation of various steps involved in the formation of Randall's plaque and development of kidney stones. Supersaturation of intratubular solute including hypercalciuria promote oxidative stress in tubular cells and epithelial to mesenchymal differentiation; cells acquire osteogenic like features and produce CaP crystals. Crystals extrude out of cells, coalesce and aggregate to form Randall's plaques at the inner medullary interstitium. Growth of plaque induces erosion through papillary epithelium into urinary lumen. Solute supersaturation, non-crystal compounds and pH of urine in will determine final stone composition.

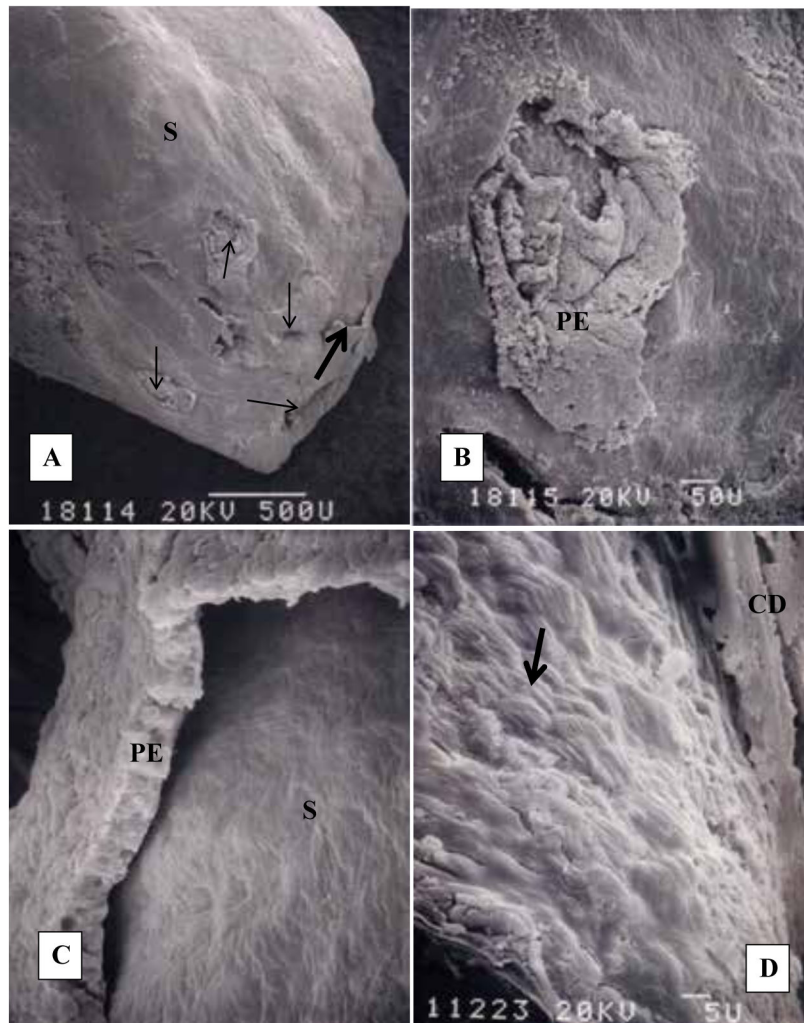


Figure 8. Human renal papilla with Randall's plug. A). CaOx stone (S) surface is exposed with the loss of epithelium. Thin arrows point out the openings of the ducts of Bellini. B). Highly magnified area showing one of the ductal openings. Part of the surface epithelium (PE) is still intact. C). Highly magnified area of A. pointed out by a single thick arrow. Surface epithelium (PE) is pulling away from the underlying stone (S). D). Stone surface showing tips of monoclinic plate like crystals of CaOx monohydrate. Adapted from Khan and Canales, 2015, *Urolithiasis* 43 Supplement 1:109–123 (10).

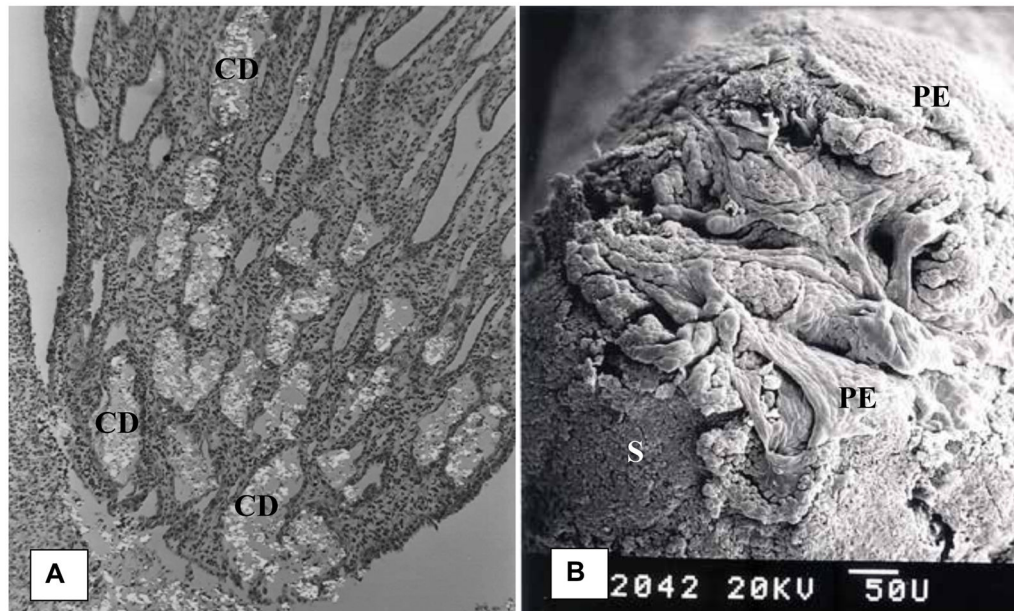


Figure 9. Papillary tip of a rat with hyperoxaluria. A). Light microscopic appearance of a longitudinal section showing birefringent CaOx monohydrate crystals plugging the collecting ducts (CD). B). Scanning microscopy of the papillary tip. Surface epithelium (PE) is eroding exposing CaOx deposit (S) plugging the ducts of Bellini. Openings of the ducts appear impaired.