Introduction
1. INTRODUCTION

Kidney stones are a common problem occurring between 30-60 years of age and continue to occupy an important place in urological practice. It emerged as a health problem in Western countries at the beginning of the 20th century; indeed, its prevalence is increasing. They are nothing but hardened mineral deposits that form in the kidney. They originate as microscopic particles and develop into stones over time. They can be present in the ureters, bladders as well as in the kidneys and vary in size from a spec of salt to the proportions of a golf ball. Kidney stone formation is a multifactorial disorder resulting from metabolic abnormalities influencing the composition of body fluids and urine. Small calculi are usually passed outside the body along with the urine stream and it becomes extremely painful only when larger stones obstruct the flow of urine going out. Clinically, urolithiasis (urinary calculi or stones) refers to calcifications that form in the urinary system, primarily in the kidney (nephrolithiasis) or ureter (ureterolithiasis), and may also form in or migrate into the lower urinary system (bladder or urethra) (Figure 1.1).

1.1. Incidence and prevalence

Urinary tract stones have been documented historically as far back as the Egyptian mummies. Kidney stones affect a significant proportion of the population, with a lifetime risk of passing a stone being about 8-10% (Asplin et al., 1996). Increased incidence of kidney stones in the industrialized world is associated with modern standards of living and is strongly associated with race or ethnicity and region of residence (Stamatelou et al., 2003). Every year, about 2-5% of the population in Asia, 8-15% in Europe and North America, and 20% in Saudi Arabia develop renal stones in their lifetime (Robertson, 1993). The
The different sites of urinary system prone to calcifications are kidney, ureter and bladder.
prevalence and incidence of stone formation in Asian countries such as India, Taiwan and Japan has been increasing in recent years. In India, there is an increased incidence of kidney stones in Rajasthan particularly in Udaipur and in some parts of North Western India (Pendse et al., 1984; Pendse and Singh, 1986). Studies carried out in our laboratory during the past thirty five years in Tamil Nadu have shown that parts of Vellore, Chengelpet, Kancheepuram, Neyveli and central districts have an increased incidence of stones which is attributed to the mineral content of water, temperature and humidity. The increased incidence of urolithiasis over the past century can be attributed to genetic, nutritional and environmental factors, changes in life style and dietary pattern. In the majority of patients, the symptoms and consequences are not life threatening, but stones in the urinary tract are a major cause of morbidity, hospitalization and days lost from work. The estimated total annual cost for treating stone disease is $1.83 billion in the United States (Chandhoke, 2002), £111.3 million in the United Kingdom (Robertson, 1999a) and 54.38 million Euro in Germany (Strohmaier and Hormann, 2000).

Among the various types of stones, about 80% are composed of calcium salts and usually occur as calcium oxalate and less commonly as calcium phosphate (Daudon et al., 1995). The remaining 20% of stones are composed of uric acid, struvite or carbonate apatite, cystine and rare stones. “Infection” stones are of struvite or carbonate apatite and have become less common with the introduction of antibiotic therapy. Uric acid stones, which account for 5-10% of stones in USA and Europe, occur more frequently in patients in certain near-Eastern and Mediterranean countries (30%). Cystine stones comprise roughly 1% of stones.
1.2. Types of stones

The chemical composition of stone depends on the chemical imbalance in the urine. Based on the urinary constituents, calculi is divided into

a) Simple calculi, which contain a single urinary constituent

b) Mixed calculi, which contain two or more substances present in urine

c) Foreign body calculi, which may be present due to introduction of some substance from outside

Once the stone is removed, the chemical composition of the stones are analyzed to understand the biochemical abnormality underlying stone formation. The four most common types of stones are calcium, uric acid, struvite and cystine.

i) Calcium stones

Calcium stones are the most common type of stones resulting in kidney obstruction and constitute approximately 80% of all cases. Calcium usually combines with oxalate or phosphate, resulting in either calcium oxalate or calcium phosphate stones.

a. Calcium oxalate

Calcium oxalate is the most frequent constituent found in urinary calculi and consist of approximately 50% oxalate, 30% calcium, 9% phosphorus and 4.5% uric acid. There are two types of hydrates of calcium oxalate, calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD). COM, CaC₂O₄·H₂O stones are porous or granular types of structures occurring in the calculi of mixed composition. COD, CaC₂O₄·2H₂O consist of either loosely
intergrown aggregates of individual crystals or compact interlocking aggregates with a coarse crystal surface.

b. Calcium phosphate (Brushite)

The only simple calcium phosphate existing in calculi is calcium hydrogen phosphate dihydrate (CaHPO$_4$ 2H$_2$O), which is fibrous and silky white in appearance. Calcium phosphate stones are enriched with phosphorus (52%), calcium (29%), oxalate (12%) and uric acid (1%). Calcium phosphates typically occur in patients with metabolic or hormonal disorders such as renal tubular acidosis and hyperparathyroidism (Williams et al., 2001).

ii) Uric acid stones

Uric acid stones (C$_3$H$_5$N$_4$O$_3$) comprise approximately 5-10% of stones and is typically smooth, round, yellow, orange and radiolucent. Uric acid calculi are composed of 45% uric acid, 20% calcium and 19% oxalate. Hyperuricosuria is the sole metabolic abnormality in 15-20% of patients with recurrent calcium oxalate stones, and treatment of this subpopulation of hyperuricosuric calcium oxalate stone formers with allopurinol, to reduce urinary uric acid excretion, reduces subsequent formation of stones (Ettinger et al., 1986).

iii) Struvite calculi

Struvite stones are also called as Staghorn calculi and are made up of magnesium ammonium phosphate. Urinary infection with urea splitting organisms (Proteus, Klebsiella, Serratia and Mycoplasma) creates alkaline urine that promotes the formation of struvite stones.
Apart from these major types of stones, cystine, xanthine and other miscellaneous stones are also found, which are of minor importance.

1.3. Epidemiological and aetiological factors favoring stone formation

1.3.1. Epidemiological factors

These factors are divided into intrinsic and extrinsic factors.

1.3.1.1. Intrinsic factors

Intrinsic factors include genetic, racial and any inherited physiologic or anatomic predisposition to urinary calculi. Genetic factors have been postulated to play an important role in urolithiasis and are supported by the evidence that positive family history is a well known risk factor for urolithiasis (Resnick et al., 1968; Goodmann et al., 1995). Lee et al. (2002) have also clearly demonstrated the importance of family history for idiopathic calcium stone formation. However, a clear picture of genetic contribution to urolithiasis is hampered by its multifactorial nature. The anatomy of the upper and lower tracts may also influence the likelihood of stone formation by predisposing to urinary tract infection or stasis. Urinary stones occur more often in white populations than in black populations, indicating that race also plays an important role in stone formation. Sex plays a major role in determining the susceptibility for stone formation. In males, the increased secretion of testosterone results in increased endogenous oxalate production by the liver (Richardson. 1967; Kuczera et al., 1993). Hence, they are at a greater risk than females, with a male-to-female ratio of 3:1 (except for struvite stones and in black populations). Incidence of stones also increases with age and the peak age for development is 30-60 years.
1.3.1.2. Extrinsic factors

Geography has an effect in terms of temperature and humidity, which seems to influence the incidence of human urinary calculi. The rate of prevalence is higher in those who live in mountains, deserts and tropical areas like United states, Scandinavian, Mediterranean countries, India, Pakistan, Australia, Central Europe, portions of Malayan peninsula and China. Geographical location of a place influences stone formation through its effect on temperature and season. The hot climate contributes to dehydration with decreased urinary volume, increased urine osmolality and increased concentration of calcium and oxalate. Apart from geography and climate, one’s personal habits like fluid intake and diet also greatly influence stone formation. The seasonal variations also affect the dietary intake. A careful observation of dietary history is critical for the evaluation, as intake of various foods and fluids, which result in greater urinary excretion of substances that produce stones, has a significant effect on the incidence of urinary calculi. Urinary diuresis reduces the average time of residence of free particles in urine and dilutes the components of urine that may crystallize. Increased fluid intake decreases the supersaturation of urine and subsequently the propensity to stone formation. Moreover, reports show that the incidence of urinary calculi is higher in administrative and sedentary personal, cooks and engineering room personals, suggesting that occupation of a person could have some influence on stone formation. Certain nephrotoxic effects of hot metal fumes also increase the incidence of kidney stones (Ferrie and Scott, 1984).
1.3.2. Aetiological factors

Hypercalciuria, hyperoxaluria, hyperuricosuria, hypocitraturia and renal tubular acidosis are important aetiological factors influencing stone formation. Hypercalciuria is defined as the excretion of more than 4 mg of calcium/kg body weight/day, or more than 7 mM in men and 6 mM in women. Hypercalciuria contributes to calcium stone formation by increasing the relative supersaturation of urine and by forming a complex with anions such as oxalates or phosphate. However, a factor that had been overlooked for a long time is that urinary excretion of oxalate is 15 times more potent in terms of urinary supersaturation than urinary calcium (Robertson and Peacock, 1980). Indeed, a simple chemical experiment demonstrated that only a small quantity of sodium oxalate was required to initiate spontaneous crystallization, whereas it was almost impossible to initiate crystallization by adding calcium chloride (Robertson, 1999b). Thus, urinary oxalate is now correctly regarded as the limiting risk factor, rather than urinary calcium. Increased dietary intakes, intestinal hyperabsorption, increased endogenous production of oxalate, deficiency of oxalate-degrading bacteria are considered as the important mechanisms which increase urinary oxalate (Robertson, 1999b). Increased amount of uric acid (hyperuricosuria) is also relatively common among stone formers (Farell et al., 2004). Hyperuricosuric nephrolithic patients typically excrete more than 600 mg uric acid/day, and have an urinary pH greater than 5.5, with the dissociated form of uric acid predominating in urine. Supersaturation of urine with respect to monosodium urate can directly induce the heterogeneous nucleation of calcium oxalate (Farell et al., 2004). Hypocitraturia is reported in upto 50% of patients with calcium oxalate nephrolithiasis. Citrate has inhibitory activity with respect to calcium oxalate (and calcium phosphate) crystallization, aggregation and agglomeration, such that low urinary levels can predispose to calcium oxalate urolithiasis (Laube
et al., 2002). Citrate complexing with calcium in the urine inhibits spontaneous precipitation of calcium oxalate crystals (Parks et al., 1996). Renal tubular acidosis is a term applied to several conditions in which metabolic acidosis is caused by specific defects in renal tubular H⁺ gradient by the distal tubule. Inherent defect in excreting H⁺ significantly reduces urinary citrate and can lead to stone formation, usually calcium phosphate.

1.4. Diagnosis

Diagnosis of renal stone disease involves a medical history, physical examination, laboratory evaluation and imaging tests. The physician verifies if the patient has a history of kidney stones, documents past medical conditions, and evaluates the present symptoms. Laboratory tests include urine analysis to detect the presence of blood (hematuria), bacteria (bacteriuria) in the urine, blood tests for creatinine (to evaluate kidney function), blood urea nitrogen (BUN) and electrolytes (to detect dehydration), calcium (to detect hyperparathyroidism), and a complete blood count (CBC; to detect infection). Apart from the routine analysis, imaging tests such as ultrasound, intravenous pyelogram, retrograde pyelogram, and computerized tomography scan are used to diagnose stones. Ultrasound tests use high-frequency sound waves to produce pictures of internal structures (e.g. organs, kidney stones). Ultrasound can also detect a dilated (stretched) upper urinary tract and kidney caused by a stone lodged in the ureter, but usually cannot detect small stones, especially those located outside the kidney. But, it is the preferred imaging method for kidney stone patients who are pregnant. Intravenous pyelogram test involves taking a series of X-rays after injecting a contrast agent (dye) into a vein. The contrast agent flowing through the veins, excreted by the kidneys, improves the X-ray images of kidneys and ureters. Most kidney stones (e.g. calcium stones) can be precisely located using
this procedure. There is a slight risk for an allergic reaction to the contrast agent during this procedure and overall kidney function must be normal. Retrograde pyelogram is a cystoscopy (i.e. a procedure in which a telescopic instrument is inserted into the urethra) and is performed to locate the opening from the ureter to the bladder. The contrast agent is injected directly into this opening and an X-ray is taken to locate the kidney stone. The retrograde pyelogram is the most reliable method for visualizing the urinary system and detecting stones, it is generally used only when other imaging methods are inadequate or unsuccessful (Figure 1.2). Computerized tomography uses a scanner and a computer to create images of the urinary system. It is performed quickly but may have difficulty detecting small stones located near the bladder. Computerized tomography scan also helps to identify medical conditions (e.g., ruptured appendix, bowel obstruction) that cause symptoms similar to kidney stones. The non-contrast computerized tomography scan is the most common imaging test used to evaluate a kidney stone. If any stone is found, a plain abdominal X-ray is also taken to determine their size, shape and orientation. X-rays are used for follow-up studies to monitor the progress of stones.
Figure 1.2. Diagnosis of kidney stones using retrograde pyelogram

Dye is injected, and an X-ray is taken
1.5. Lithotriptic techniques to treat stones

Treatment of a kidney stone depends on its size, location, composition, presence of anatomical malformation and complications. The indications for surgical intervention for upper tract stones include recurrent pain, high-grade obstruction associated infection, and large size of stones. Treatment options include shock wave lithotripsy, ureteroscopy, extracorporeal shock wave lithotripsy, percutaneous nephrolithotomy and open or laparoscopic stone removal. Shock wave lithotripsy is the most commonly employed treatment modality for renal and ureteral calculi and for stones associated with some anatomic abnormalities, specifically obstruction (e.g. ureteropelvic junction obstruction, ureteric stricture, etc.). Ureteroscopy is primarily used to treat ureteral stones, but is increasingly being used to treat renal calculi for which shock wave lithotripsy has failed or is ill-advised. Percutaneous nephrolithotomy is indicated for large-size renal calculi and for stones associated with some anatomic abnormalities. Finally, open and laparoscopic surgery are reserved for stones that are not treatable with less invasive treatment options or are associated with extensive anatomic abnormalities that require simultaneous repair. However, open or laparoscopic therapy for urolithiasis is indicated in fewer than 2% of patients today. Bladder stones are predominantly treated with endoscopic fragmentation, and less commonly with shock wave lithotripsy or open procedures.

1.5.1. Need for a prophylactic approach

Advancements in lithotriptic techniques have improved the standard of living of stone formers. However, disadvantages associated with these techniques like a high recurrence rate, hemorrhages, residual stone fragments limit the usage
of these techniques. Moreover, the accepted stone recurrence rate for all patients is approximately 50% over 10 years and this is not a trivial recurrence rate from patients perspective, considering the morbidity, cost of treatment to the individual, cost of lost work time and not withstanding the severe pain associated with renal colic. The major risk factors for recurrence are suggested to be male sex, multiple and lower calyx stones, early onset, familial history and complications after stone removal (Strohmaier, 2000). The high recurrence rate emphasizes that stone formation is a consequence of a complex physico-chemical event fuelled by molecular disturbances. Hence, removal of the stones with lithotriptic techniques, without modulating the underlying molecular disturbance increases the recurrence of stones.

Thiazide therapy is indicated for renal hypercalciuria, but it is not ideal for absorptive hypercalciuria. Orthophosphate would seem to be the logical treatment for hypophosphatemic absorptive hypercalciuria because of its inhibition of 1,25-dihydroxy cholecalciferol synthesis. Allopurinol has been reported to be of therapeutic benefit for hyperuricosuric calcium oxalate nephrolithiasis. Low urine volume promotes idiopathic calcareous stone formation and hence, patients are advised to drink more water (Pak et al., 1980). Other dietary measures advised are to decrease the consumption of sugar, ascorbic acid, purine (Coe and Kavalach, 1974), fat (Perrochet et al., 1981) and animal protein (Fellstrom et al., 1983). Inhibition of aldehyde oxidizing enzymes with disulphiram and acetomenaphone was able to block formation of glycollate and hence effective in preventing crystallization. Pridoxine supplementation was also able to prevent hyperoxaluria (Kasidas and Rose, 1984). Magnesium oxide therapy has been reported to be of therapeutic benefit in patients with primary hyperoxaluria (Silver and Brendler, 1971). Colestipol and aluminium hydroxide administration reduced dietary oxalate absorption in patients with
enteric hyperoxaluria (Laker and Hoffmann, 1981). Fry and Richardson (1979) have evaluated the inhibitory effects of some mono and dicarboxylic acids such as malonate and succinate on endogenous oxalate synthesis. Potassium citrate therapy was also effective to a certain extent in curtailing lithogenesis.

1.5.2. Biomolecules tested against calcium oxalate crystallization in our laboratory

Our laboratory has been actively engaged in kidney stones research for the past three decades, and has explored the efficacy of numerous synthetic and naturally occurring molecules potencies, to modulate stone formation. Selvam and Varalakshmi (1990a,b and 1991) have elucidated the beneficial role of isomers of tartarate on oxalate metabolism in rats and their inhibitory efficacies in vitro by calcium oxalate crystallization. DL α-Lipoic acid is a universal antioxidant, with a potential to modulate many pathologies resulting from oxidative injury. The effect of lipoic acid on peroxidative and membrane injury was studied in stone forming rats by Jayanthi and Varalakshmi (1992) and Sumathi et al. (1994 and 1995). Studies on the efficacy of eicosapentaenoic (EPA), a polyunsaturated fatty acid found in fish oil and its derivative with lipoic acid (EPA-LA) in preventing calcium oxalate crystallization has been carried out by Lenin et al. (2001 and 2002). The protective efficacy of L-cysteine, a thiol replenishing agent against calcium oxalate crystallization was investigated by Saravanan et al. (1996). Numerous phytodrugs are known to possess antiurilithic properties and the efficacy of the phytodrug, lupeol (pentacyclic triterpene) isolated from Crataeva nurvala in experimentally induced calcium oxalate lithiasis was elaborately studied by Baskar et al. (1996) and Malini and Varalakshmi (1998). Drugs like coleus aromaticus, ripe kernel juice of plantain (Musa paradisiaca) were tested for their efficacy in experimental hyperoxaluria.
and were found to be effective in mitigating the oxidative stress associated with hyperoxaluria (Baskar et al., 1992; Poonguzhali and Varalakshmi, 1992; Kalpana Devi et al., 1993). Phycocyanin, an important constituent of the blue green algae, reported to be a good antioxidant was tested for its efficacy against hyperoxaluria and was found to be an effective inhibitor of crystallization (Farooq et al., 2004). Sumita et al. (2005) showed the beneficial effect of vitamin E therapy on the biochemical and kinetic properties of Tamm Horsfall glycoprotein in hypertensive and hyperoxaluric patients. But, lack of a complete drug for kidney stones emphasizes that understanding the disturbances in the molecular mechanism is essential to develop a complete therapy for stones.

1.6. Molecular derangements and stone formation

Recent research in the field of urolithiasis strongly suggests that molecular derangements undoubtedly are essential for the progression of stone disease (Jonassen et al., 2003). Chen et al. (2004) have also demonstrated that changes in renal phenotype especially alterations in the expression of genes associated with inflammation, oxidative damage, tubule function and regulation, were the most common manifestations in the functional categories in ethylene glycol model of rat urolithiasis. Large body of evidences emphasize that aberrant change in molecular events act as a trigger for stone formation. Hence, a clear picture underlying molecular disturbance is necessary to curtail stone formation.

The forthcoming literature highlights the possible mechanisms put forward by various researchers to explain stone formation. With respect to this, many theories have been proposed for the pathogenesis of urolithiasis including matrix nucleation theory, precipitation crystallization theory, inhibitor absence theory, fixed particle retention theory and injury induced crystal retention theory.
Of these, injury induced crystal retention has received considerable attention in the recent years due to the renewed interest and understanding of the role of free radicals in urolithiasis. The theory proposes that crystal retention is a self-perpetuating cycle, where injured urothelium might either nucleate crystals or preferentially attach preformed crystals. The crystals would then further damage the cells to which they are attached, generating more injured tissue that complete the cycle by inducing more crystal retention, either by nucleation of new crystals or by attachment of crystals formed higher in nephron.

1.6.1. Injury to the renal tubules by free radicals

Free calcium oxalate crystals formed within the renal tubule cannot grow rapidly enough to block a collecting duct at the rate of normal urinary flow to form a kidney stone, because the time needed for a crystal to grow to a diameter of 200 μm and block the nephron is calculated to be from 90 min to 1500 years (Finlayson, 1974). Crystals are known to adhere to damaged bladder urothelium but not to the healthy tissue, and therefore molecular adhesion and stagnation of crystals in an anatomically constrained region play a vital role in the pathogenic mechanism of stone formation (Khan et al., 1984; Verkoelen et al., 1997, Lieske et al., 2000). The role of renal injury in stone formation is further strengthened by the observation of elevated levels of renal tubular cell derived enzymes and cytokines in urine of recurrent stone-formers (Baggio et al., 1983; Rhee et al., 1998).

Research has identified that injury to the tubules is mediated by the free radicals produced by oxalate, one of the important constituents of stone formation (Farooq et al., 2004; Huang et al., 2003a). It is a dicarboxylate byproduct of metabolism, freely filtered at the glomerulus, where its
concentration is normally around 1-5 μM, but at the cortical collecting duct, it may reach >300-500 μM, with even higher concentrations observed in patients with primary hyperoxaluria (Asplin, 2002). Many laboratories have demonstrated that exposure to oxalate at these concentrations can elicit a range of toxic responses at the level of renal cells. Accumulation of hydroperoxides and decreased free radical scavengers have been demonstrated in hyperoxaluric rats (Farooq et al., 2004).

Oxalate-induced cell injury is a result of free radical-induced stress-activated protein kinase-mediated inflammation (Miller et al., 2000; Bhandari et al., 2002; Chaturvedi et al., 2002). Recent studies have shown that apart from oxalate, COM crystals also inflict damage to the renal tubules through free radicals production (Thamilselvan et al., 2003). The notion that hyperoxaluria produces free radical was further supported by the studies demonstrating the beneficial effects of free radical scavengers like lipoic acid and vitamin E in hyperoxaluria (Sumathi et al., 1993; Thamilselvan and Menon, 2005). Oxalate can also provoke other cellular responses varying from activation of DNA synthesis to apoptotic and necrotic cell death (Miller et al., 2000).

Recent studies have also shown that reactive nitrogen species can also mediate the same chemistry as reactive oxygen species (ROS) in stone formation. Studies strongly suggest the role of nitrosative stress in pathologies ensuing due to redox imbalance (Grisham et al., 1999). Nitrosative stress results due to the increased production of reactive nitrogen intermediates like nitric oxide, peroxynitrite, etc. Nitric oxide is a ubiquitous signaling molecule which is produced in small amounts by the constitutive, neuronal and endothelial nitric oxide synthase (nNOS and eNOS) under physiological conditions (Grisham et al., 1999). Transient spike-like generation of nitric oxide characteristic of
eNOS activation is critical for turning on several biologically important events. However, sustained high generation of nitric oxide by the inducible isoform (iNOS) is deleterious and may turn on a broad spectrum of sequelae from pro-apoptotic effects to LPO and DNA damage. Pragasam et al. (2006) have shown that nitrosative stress plays an important role together with oxidative stress in bringing about the pathological effects of oxalate in hyperoxaluria. Increased production of nitric oxide is known to reduce the inhibitory effect of Tamm Horsfall glycoprotein (Pragasam et al., 2006). Oxidative stress associated with cerebral ischemia and myocardial reoxygenation increases nitric oxide production through iNOS, but the mechanism mediating induction of iNOS after exposure to oxidant stress remains unknown (Malinsky et al., 1993; Morita et al., 1994). However, both oxygen and nitrogen based reactive intermediates are known to have detrimental consequences, hence it becomes essential to identify their sources and initiate measures to limit their production in the cell within limits.

1.6.1.1. Mitochondrial dysfunction and renal tubular injury

Redox imbalance resulting from increased production of reactive intermediates is known to initiate diverse cellular effects ranging from upregulation of key transcription factors, gene induction, cell proliferation, etc. (Hensley et al., 2000). Although studies support the role of reactive species in oxalate induced toxicity, little is known of the origin or the role of reactive intermediates in oxalate-cell interactions. Mitochondria, as sites of aerobic metabolism, are likely to be a significant source of intracellular ROS (Thannickal and Fanburg, 2000). Under physiological conditions, ROS produced is well within cells limit, for the aerobic organisms have evolved an impressive array of antioxidant defense system. In pathological conditions, interruption of electron
transport chain due to mitochondrial injury or dysfunction contributes to ROS accrual by mitochondria (Fleury et al., 2002).

Khand et al. (2002) suggested that mitochondria mediated superoxide production is an important contributor of oxidative stress in renal epithelial cells under oxalate overload. They showed that non-mitochondrial superoxide inhibitors failed to abrogate the production of superoxide on incubating Madin-Darby Canine Kidney (MDCK) cells with COM crystals. By comparison, mitochondrial inhibitors of complex I and II blocks the flow of electrons towards complex III and thereby inhibited superoxide formation. McMartin and Wallace (2005) demonstrated that COM inhibited State 3 respiration in rat kidney mitochondria. Mitochondrial swelling induced by COM was completely blocked by cyclosporine A, indicating that COM-induced mitochondrial alterations is mediated by the opening of mitochondrial permeability transition pore.

Mitochondrial dysfunction apart from producing reactive species can also activate apoptotic events, as mitochondria is known to play a crucial role in the intrinsic pathway of apoptosis. Miyazawa et al. (2005) have shown an induction of apoptosis-related genes including Bax in renal epithelial cells of stone-forming rats. Increase in apoptosis on oxalate exposure increases the incidence of stone formation several fold, as it exposes the basal lamina favoring crystal adherence. The increase in oxidant stress that accompanies oxalate-induced changes in mitochondrial function appears to be responsible for many of the toxic effects of oxalate on renal cells like decreased membrane integrity, redistribution of membrane phospholipids, increased cell death via apoptosis and necrosis, generation of lipid peroxides and release of cellular enzymes (Jonassen et al., 2003).
1.6.1.2. Inflammation and renal tubular injury

Recent studies on renal morphology in hyperoxaluric condition have shown that crystal deposition is accompanied by an infiltration of immune cells. Moreover, necropsy studies of human kidneys have revealed distinct signs of injury, inflammation including necrosis of tubular epithelium and capillary endothelium along with the presence of myofibroblasts, a large amount of collagen and large numbers of leukocytes in the interstitium (Khan, 2004). In response to hyperoxaluric conditions, there is an influx of cells expressing the leukocyte common antigen (CD 45), the ED1 antigen and the major histocompatibility class II antigen into the kidney (Khan, 2004).

Inflammatory cells such as monocytes, macrophages and polymorphonuclear leukocytes migrated to the adjacent interstitium and interstitial crystals were often seen surrounded by macrophages and giant cells (Khan and Thamilselvan, 2000). Studies by de Bruijn et al. (1995a) also showed that in experimental calcium oxalate nephrolithiasis, crystals form in the tubular lumen and eventually move into the interstitium causing inflammation and attracting many inflammatory cells. These cells may play an important role in renal tissue damage through the production of proteolytic enzymes, cytokines, and chemokines (Muller and Rodemann, 1991). The mechanism by which inflammatory cells enter the renal interstitium is not known, but it is likely that chemotactic factors and adhesion molecules are involved.

Khan (2004) has suggested that renal epithelial cells, in response to crystal exposure produce chemokines, which attract the macrophages/monocytes towards the sites of crystal deposition. Increased expression of monocyte chemoattractant protein-1 (MCP-1) have been reported in renal epithelial culture,
on exposure to calcium oxalate, thus supporting the role of cytokines in mediating the inflammation process in urolithiasis (Umekawa et al., 2003). MCP-1 is a member of the cysteine-cysteine (CC) chemokine subfamily and is chemotactic for monocytes and other leukocyte subsets with a potential to elicit an inflammatory and immune reaction. Most chemokines can be produced by a wide variety of cell types after proper stimulation with proinflammatory cytokines, or even by direct stimulation. Further, increase in MCP-1 is also mediated via the ROS as addition of catalase was able to abrogate the expression of MCP-1 (Umekawa et al., 2003).

Increase in ROS might also trigger inflammatory reaction as it activates many signaling molecules such as protein kinase C, c-Jun N-terminal kinase, p38 mitogen-activated protein kinase and transcription factors such as nuclear factor-kB (NF-kB) and activated protein-1 (AP-1) (Khan, 2004). Activation of these molecules in turn leads to up-regulation of genes and proteins such as MCP-1, osteopontin, fibronectin and transforming growth factor-β1 (TGF-β1). TGF-β1 has a central role in regulating renal fibrosis and increasing extracellular matrix (ECM) synthesis during lithogenesis. Numerous studies have reported that TGF-β1 inhibits matrix degradation, regulates type I, type III, and type VI collagen synthesis and also participates in apoptosis (Toblli et al., 1999). Increase in collagen content and TGF-β1 expression has also been reported in hyperoxaluria (Toblli et al., 1999). Increased ECM synthesis remarkably alters the lithogenic process by tissue remodeling which exposes crucial crystal binding molecules favoring crystal retention (Toblli et al., 1999; Umekawa et al., 2004).

Other studies have provided evidence for the activation of the renin–angiotensin system during the development of tubulointerstitial lesions of
Figure 1.3. Hyperoxaluria and calcium oxalate stone formation

Hyperoxaluria and calcium oxalate crystal formation in renal tubules

- Stress on tubular epithelial cells
  - Mitochondrial damage
  - Increase in cytokines
    - Infiltration of inflammatory cells

- Production of reactive species
- Activation of renin-angiotensin system
- Lesion formation in kidney
- Tissue remodeling
- Exposure of crystal binding molecules

Crystal attachment to tubular epithelial cells

Crystal deposition and kidney stone formation
calcium oxalate crystals (Toblli et al., 2001 and 2002). The renin-angiotensin system plays an important role because angiotensin II (Ang II) participates in key events of the inflammatory response such as vascular permeability, infiltration of inflammatory cells, and tissue repair and remodeling. Various actions of Ang II and TGF-β1 are mediated by oxidative stress. Moreover, regulation of NF-κB, a nuclear transcription factor which is intimately involved in inflammation, is also redox dependent. These reports strongly suggest that inflammation and ROS can be considered as the major determining factors of stone formation and therapeutic strategies targeting them would be beneficial in urolithiasis. The literature review have been summarized in Figure 1.3.

1.7. Glycosaminoglycans-modulators of stone formation

The formation of urinary stones can be explained by the physical and chemical properties of crystals, supersaturation and lack of inhibitors. In the pathogenesis of urolithiasis, a pivotal role has been attributed to the presence of inhibitors in the urine, which decreases supersaturation, necessary to form a crystalline nucleus and prevent its growth once formed. Thus, any imbalance in the inhibitors of crystallization can lead to nucleation, growth and aggregation of crystals (Robertson et al., 1976). Inhibitors of crystallization can be divided into two classes: small molecules (citrate and pyrophosphate) and macromolecules (glycoproteins and glycosaminoglycans [GAGs]) (Worcester, 1996). GAGs are polysaccharide chains composed of repeated identical disaccharide units. Each of these units contains two monosaccharides, a hexosamine and other an N-free monosaccharide, mostly glucuronic acid. GAGs are ubiquitously distributed in mammalian tissues and are found as proteoglycan complexes at cell surfaces (Hook et al., 1984). In the course of proteoglycan metabolism in the tissue, the peptide chains disintegrate proteolytically enabling the fragments to leave the
tissue. Only 10% of GAGs obtained from degradation of proteoglycans is excreted in the urine, the remainder is decomposed by lysosomal enzymes (Hesse et al., 1991). Six types of GAGs are present in the urine: heparan sulfate, chondroitin sulfate A, chondroitin sulfate C, dermatan sulfate, hyaluronic acid, and keratan sulfate (Angell and Resnick, 1989). In adults, approximately 250 mg of GAGs are metabolized each day and about 10% of the metabolites are excreted in urine.

The role of GAGs in the development and regulation of lithiasis continues to perplex and challenge investigators in the field of stone research. Interest in GAGs as important contributors to the regulation of stone formation and growth had its origin from early in vitro studies, wherein commercial preparations of certain GAGs were found to be powerful inhibitors of calcium oxalate crystallization (Robertson et al., 1973). In vivo studies also showed that there was a significant difference in GAGs excretion between stone-formers and non-stone formers (Grases et al., 1994; Vidhya and Varalakshmi, 2000). Due to the reduction of GAGs in the urine of patients with idiopathic calcium lithiasis, a meager amount is available to prevent calcium oxalate crystal growth and aggregation and hence, reduced inhibitory activity is determined in the urine (Cao et al., 1997a). Further support for the inhibitory role of GAGs came from the study of Parsons et al. (1990) demonstrating that the GAG layer covering the epithelium in the bladder, acts as a permeability barrier, protecting against bacterial and crystal adherence. Damaging this layer with hydrochloric acid or detergent Triton X-100 resulted in a marked increase in crystal adhesion whereas application of exogenous GAG restored the damaged GAG layer and reduced crystal adherence (de Bruijn et al., 1995b). GAGs inhibitory effect results from the competition with oxalate ions to complex with calcium ions. since GAGs are polyanionic.
GAGs also exert their inhibitory effects by preventing crystal retention, reducing urinary oxalate excretion and protecting renal tissue (Cao et al., 1997a). They are also known to play an important role in crystal-cell interactions, which is a crucial step in lithogenesis. Pretreatment of calcium oxalate crystals with sulfated GAGs reduced their adherence to MDCK cells in culture (Verkoelen et al., 1996). Shirane et al. (1999) showed that dermatan sulphate and chondroitin sulphate were able to inhibit the growth of calcium oxalate crystals. Low molecular weight dextran sulphate was able to prevent calculogenesis in experimental animals (Tostes et al., 2004). Heparin was found to inhibit the endocytosis of calcium oxalate crystals in BSC-1 monkey kidney epithelial cells through an interaction with cells and not with crystals (Lieske and Toback, 1993). These observations support the hypothesis that cell-surface GAGs provide a protective barrier against crystals and oxalate ions. Iida et al. (1997) implied that calcium oxalate nephrolithic conditions might induce increased expression of heparin sulphate proteoglycan in the tubular epithelial cells and therefore was able to protect the epithelial surface. In rat remnant kidney model, tubulointerstitial damage and glomerular sclerosis were effectively circumvented with heparin sulphate administration and can be attributed to its anti-proliferative and anti-inflammatory effects (Barsotti et al., 1999).

1.7.1. Research in our laboratory with glycosaminoglycans

GAGs have been the molecule of interest for many decades in numerous laboratories due to their potential role in stone formation. Sodium pentosan polysulphate (SPP) is a semisynthetic polysaccharide and a heparin analog, which is known to have lesser anticoagulant activity. Our laboratory studies have shown that SPP, due to its anti-crystallizing effect was able to modulate the crystallization process (Senthil et al., 1996). Further, in vivo studies also added
support to the protective role of SPP against crystallization. The heparin analogue was able to decrease the excretion of stone forming constituents in urine (Subha and Varalakshmi, 1993; Senthil et al., 1998). The increase in stone forming index was normalized with SPP administration. SPP was also able to modulate the oxalate synthesizing enzymes like glycolic acid oxidase (GAO) (Subha and Varalakshmi, 1992). Increase in membrane components in urine such as protein and lipids promotes stone formation and SPP was found to normalize the abnormal lipid metabolism in hyperoxaluric rats (Subha et al., 1992a).

Low molecular weight heparin (LMWH), another synthetic heparin analogue also proved to have reno-protective effects through our laboratory research. Our research showed that LMWH decreased ROS production and increased the antioxidant status of the renal tissues in nephrotoxic condition (Deepa and Varalakshmi, 2003a). It was also able to restore the thiol level altered during nephrotoxicity. LMWH decreased the elevation in nitric oxide level, emphasizing its beneficial effect against nitrosative stress (Deepa and Varalakshmi, 2006a). Moreover, it was able to prevent DNA damage and restore the cellular architecture (Deepa and Varalakshmi, 2006a).

Efficacy of the LMWH against injury-induced crystal retention was also tested in our laboratory. LMWH was able to protect the renal tissues from oxidative stress (Rajeswari and Varalakshmi, 2006). Administration of LMWH to gentamicin and ammonium chloride administered rats not only reduced the renal damage but also prevented crystal retention. Histopathological analysis of the renal tissues revealed that LMWH effectively prevented renal damage. Tubular uptake of crystals was prevented by the LMWH administration in hyperoxaluria. Further, they have shown that LMWH was able to decrease the fibrosis of kidney, indicating that LMWH can influence the ECM synthesis.
Since, heparin and its analogues were found to be effective in preventing lithogenesis, it can be hypothesized that compounds bearing similarity with heparin would also be positive modulators of lithogenesis.

1.8. Fucoidans from brown algae-naturally occurring glycosaminoglycans

Fucoidan, the major cell wall GAGs of brown algae bears similarity with heparin (Shanmugam and Mody, 2000). Fucoidan constitutes about 4% of the total dry weight of many types of brown seaweed. It was first isolated from marine brown algae 90 years ago (Kylin, 1913) and later, it was also found to occur in marine invertebrates (Vasseur, 1948). Fucoidan is a sulphated polysaccharide that possesses a complex structure and is an important component of membrane in marine algae and in egg jelly coat of marine invertebrates. Its chief constituents include a sulfuric acid esterified L-fucose, and traces of galactose, xylose, and glucuronic acid. Algal fucoidans are present in several orders, mainly Fucales and Laminariales and also in Chordariales, Dictyotaales, Dictyosiphonales, Ectocarpales and Scytosiphonales. They are widely present in all the brown algae (Phaeophyceae) and seem to be absent from green algae (Chlorophyceae), red algae (Rhodophyceae), freshwater algae and terrestrial plants. Little is known about the role of fucoidans in marine organisms. In marine invertebrates, they may have a role in maintenance of the body wall integrity (Ribeiro et al., 1994). In algae, some studies have shown a correlation between fucoidan content and the depth at which they grow. The closer algae are to the surface, the greater the fucoidan content (Evans, 1989). Furthermore, fucoidans appear to play a role in the algal cell wall organization (Bisgrove and Kropf, 2001) and could be involved in the cross-linkage of alginate and cellulose (Mabeau et al., 1990). Fucoidans may also be involved in the morphogenesis of algae embryos (Bisgrove and Kropf, 2001).
Percival and Ross (1950) described fucoidan from the common brown algae *Fucus vesiculosus* as a polysaccharide based on L-fucose with mainly α(1 → 2) glycosidic bonds and sulfate groups at position 4. This model was supported by further studies from other investigators (O'Neill, 1954; Cote, 1959). They also found branches of sulfated fucose every five units. This was the only structural model available for fucoidan for more than 40 years, even though fucoidan from various algae was commonly used in biological studies. Patankar et al. (1993) reinvestigated the structure of fucoidan from *F. vesiculosus* (the only one commercially available) and proposed that the nature of the main glycosidic bond was α(1 → 3) instead of α(1 → 2) from the previous studies (Figure 1.4). To date, no fucoidan without sulphate groups have been identified, hence they can be also be designated rightly as sulphated polysaccharides.

**Figure 1.4. Structure of fucoidan from *Fucus vesiculosus***

![Structure of fucoidan](image)

The single largest source of fucoidan is brown algae. Brown algae are classified either in the Division Chromophyta or in Phaeophyta. The brown algae usually grow in large colonies giving the appearance of weeds hence they are also called as seaweeds. The color of brown algae may vary from dark brown to golden brown and even to olive green. The brown algae consist of a stalk called a stipe with flat blades called lamina branching from it. The holdfast is at the base
of the stipe which functions to hold the algae to the rock instead of being washed away. This is especially useful when the algae are growing in turbulent surf zone and intertidal areas. The meristem is at the base or at blade junctions with the oldest tissue at the tip of the blade. Some algae have gas-filled floatation bladders that help to keep their large blades near the water’s surface so they can receive more light. An image of *Fucus vesiculosus*, the brown algae explored for its anti-urolithic effect is presented in Figure 1.5.

**Figure 1.5. Fucus vesiculosus**

1.8.1. Economical uses of brown algae

Algae are used in many maritime countries in industries and as a fertilizer. The major utilization of these plants as food is in Asia, where their cultivation has become a major industry. The main food species grown by aquaculture in China, Korea and Japan are Nori (Porphyra, a red alga), Kombu or Kunbu (Laminaria, a brown alga) and Wakame (Undaria, also a brown alga).
Algae as a staple item of diet have been used in Japan and China since prehistoric times. In 600 BC, Sze Teu wrote in China, "Some algae are a delicacy fit for the most honoured guests, even for the King himself." Some 21 species are used in everyday cookery in Japan, six of them since the 8th century. Seaweed accounts for some 10% of the Japanese diet and seaweed consumption reached an average of 3.5 kg per household in 1973, a 20% increase in 10 years have been found (Ingergaard, 1983). In Japan alone, the total annual production value of nori amounts to >US$1 billion, one of the most valuable crops produced by aquaculture in the world.

Historically, algae have been used for its iodine content in the medical and photography fields as well as in the manufacture of glass and gunpowder. One of the most common modern uses for brown algae is as a commercial source of algin and alginate. These substances are used in the production of ice cream, salad dressing, beer, jelly beans, latex paint, penicillin suspensions, paper, textiles, toothpastes, ceramics, and floor polish. Alginate compounds are found in the cell walls of brown algae. Alginate is also used as a food additive for domestic animals and as a base for animal health products. Alginate and alginate salts are very soluble in water and form thick fluids in solution. When formed with metal ions, alginate salts form gels in water. These compounds are also used in a wide variety of products such as paint, artificial foods, and in fruit canning. The combination of alginic acid and heavy metals are used in plastic manufacturing, making cloth, dental impressions, and oddly enough, high quality audio speakers (Simpson and Ogorzaly, 2001).
1.8.2. Biological properties of fucoidan

Most of the biologically important properties of brown algae are mainly attributed to the presence of fucoidan. Fucoidans have a wide spectrum of activity in biological systems. In earlier times, they interested the researchers mainly as a substitute to heparin due to their lesser anticoagulant activity. Fucoidan from *F. vesiculosus* has a specific anticoagulant activity of 16 U/mg, as compared with 193 U/mg for heparin (Mourao and Pereira, 1999). Due to its similarity with heparin, fucoidan shares numerous properties of heparin. Fucoidan fractions of varying molecular weight and degree of sulfation induced platelet aggregation *in vitro* (Durig *et al.*, 1997). Like heparin, fucoidan has antiproliferative effects on vascular smooth muscle cells. A fucoidan fraction from *Ascophyllum nodosum* was more active than heparin in exhibiting the antiproliferative effects (Logeart *et al.*, 1997).

The interaction between fucoidan and selectins has physiological consequences that could be therapeutically useful: for perfusion with fucoidan reduces neutrophil infiltration and myocardial injury after ischemia/reperfusion (Omata *et al.*, 1997). Fucoidans extracted from brown algae was shown to inhibit the growth of cancer cells and even cause self-destruction of cancerous cells (Itoh *et al.*, 1993). *In vitro* studies show that sulfated polysaccharides possess antimalarial property, inhibiting the invasion of free *Plasmodium falciparum* parasites into erythrocytes (Clark *et al.*, 1997). Fucoidan and low-molecular weight fucoidan, but not desulfated fucoidan, inhibit *Plasmodium berghei* development in HepG2 cells and sporozoite invasion of Chinese hamster ovary cells (Ying *et al.*, 1997). Fucoidan was also found to inhibit virus infection of cells. This has recently been demonstrated for Herpes simplex, cytomegalovirus
and human immunodeficiency virus (Hoshino et al., 1998) as well as bovine viral diarrhea virus (Iqbal et al., 2000), probably by competing with cell surface heparin sulphate for binding to the virus.

Fucoidans emerged as an important class of bioactive natural compounds, due to their sulphate content and antioxidant potential (Ruperez et al., 2002). They are found to accumulate in the kidneys when administered systemically, suggestive of their renal effects (Guimaraes and Mourao, 1997). Zhang et al. (2003a) have reported that administration of fucoidan to rats with renal failure reversed the associated complications of the disease. Further, administration of sulphated polysaccharides decreases the tubular lesions and even reverses the condition to near normalcy in diabetic nephropathy (Gambaro et al., 1994). Fucoidan exhibits anti-inflammatory property and it is mediated predominantly by the down regulation of the cytokines. It decreases the expression of IL-6 in chronic colitis by decreasing the translocation of NF-κB (Matsumoto et al., 2004).
Scope of the Present Study
1.9. **SCOPE OF THE PRESENT INVESTIGATION**

Urolithiasis is classically explained as the derangement in the process of biomineralization. Introduction of new lithotriptic techniques have revolutionized the treatment strategies for kidney stones, but have also increased their recurrence rate. Failure of lithotriptic techniques to completely eradicate kidney stones, further support the notion that molecular derangements play a major role for the progression of stone disease. Mitochondrial dysfunction and associated increase in free radicals increase renal damage, and favor crystal retention in renal tissues. Recent studies have also shown the activation of cytokines and inflammatory related genes in urolithic condition, indicating that inflammation also contributes to a significant extent for stone formation. Prophylactic approaches targeting these abnormalities would be beneficial in the treatment of kidney stones. An imbalance in inhibitors or an increase in promoters might be the initial triggering event for the molecular derangements. In idiopathic calcium lithiasis, inhibitors play a significant role and GAGs have been reported as an important inhibitor of stone formation. Stone formers have a decreased excretion of these inhibitory molecules which correlates positively with a decreased inhibitory potential of urine. A significant number of data indicate that exogenous supplementation of GAGs is beneficial in the treatment of urolithiasis. Fucoidan, a naturally occurring GAG from the brown algae is known to possess antioxidant potential. It is capable of averting renal lesions in diabetic nephropathy and inhibits chronic renal failure. Fucoidan is also shown to exhibit anti-inflammatory property through its potential to down regulate the expression of cytokines. The antioxidant potential of fucoidan together with its anti-inflammatory effect evoked a scientific interest to test its efficacy in hyperoxaluric condition.
Hence, the present study is aimed to

- Analyze the effect of fucoidan on crystallization events using *in vitro* studies
- Evaluate the effect of fucoidan on mitochondrial dysfunction and inflammation using *in vivo* studies
- Assess the effect of fucoidan on oxalate induced apoptosis in MDCK cells.
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