OPEN ACCESS

Haya: The Saudi Journal of Life Sciences

Abbreviated Key Title: Haya Saudi J Life Sci
ISSN 2415-623X (Print) |ISSN 2415-6221 (Online)
Scholars Middle East Publishers, Dubai, United Arab Emirates
Journal homepage: https://saudijournals.com

Review Article

In vitro Studies on the Formation and Growth of Urinary Stone Crystals

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DOI: 10.36348/sjls.2021.v06i10.006 | **Received:** 05.09.2021 | **Accepted:** 18.10.2021 | **Published:** 25.10.2021

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Abstract

It is a well known fact that the urinary stone crystal disease affects a considerable number of people and makes it a major socio-economic problem in the world. Several environmental factors influence the formation and growth of many crystalline materials in the biological fluids in a human urinary system. As *in vivo* experimentations have limitations, *in vitro* investigations have been made by several researchers in the past. A success in finding the environmental factors promoting or inhibiting the formation and growth of urinary stone crystals will be of immense help to the mankind. This article provides a bief account of the results obtained in this regard along with providing some information on urolithiasis and gel methods for crystal growth. The focus is made on Whewellite, Brushite and Struvite crystals as these are among the dominant and well studied ones.

Keywords: Biomineralization; Crystal formation; Crystal growth; Gel methods; *In vitro* studies; Urinary stone crystals.

1. INTRODUCTION

The kidneys, ureters, bladder and urethra are considered to be the major parts of a normal human urinary system (renal system / urinary tract), which does the blood filtration and acts as the drainage system for urine removal from the body. This urinary system regulates the pH, pressure, and volume of the blood, controls levels of metabolites, and electrolytes and excretes the body wastes (in urine form); and is under the influence of the circulatory, nervous and endocrine systems. The blood supplied extensively from the renal arteries get filtered in the kidneys (having nephrons as their functional units) and the pure blood leave the kidneys via the renal vein. The wastes (urine) formed in the kidneys (after blood filtration) exit through the ureters, then get stored in the bladder and subsequently get expelled through the urethra (by urination). The male and female urinary systems differ only in the urethra length. The amount of urine produced per day in a healthy human is 800 – 2000 ml, which depends on the kidney funcion and fluid intake. The urination process is under voluntary control in healthy humans and may occur as an involuntary reflex in infants, some elderly individuals and those with neurological injury.

The formation of stones (calculi) in the urinary system is called ‘urinary stones’ or ‘urinary lithiasis’ or ‘urolithiasis’. It is related to the decrease of urine volume or the increase of excretion of stone-forming components (calcium, oxalate, urate, cystine, xanthine, phosphate, *etc.*) [1]. Biomineralization or crystallization in the urinary system appears to take place opportunistically and more abundantly and the urinary stone disease is one of the oldest diseases of mankind. Stones formed in the kidneys, termed as ‘kidney stones’ or ‘renal stones’ or ‘nephrolithiasis’, consist mostly of crystalline materials (about 98 %) and about 50 types (many with extreme rarity) of stones have been recognized; calcium oxalate and calcium phosphate crystals are very often found, followed by struvite, uric acid, and cystine [2-8].

The urinary stone (or stone crystal) disease affects a considerable number of people in the world and it is considered to be a major socio-economic problem of the mankind. It has been found that several environmental factors influence the formation and growth of many urinary stone crystals in the biological fluids of the human urinary system. As *in vivo* experimentations have limitations, no clear understanding could be made possible about the environmental factors promoting or inhibiting the crystallization process in the urinary system. This tempted the researchers in carrying out *in vitro* investigations on the crystallization of urinary stone constituents [8-11]. Moreover, it has been proved that, in certain cases, the action of environmental factors is

similar in the *in vivo* and *in vitro* crystallization systems [8].

A success in finding the environmental factors promoting or inhibiting the formation and growth of urinary stone crystals will be of immense help to the mankind. Several researchers have worked on this and reported their results. A brief account of these results will be dealt in this article along with providing some information on urolithiasis and also on gel methods for crystal growth, as the *in vitro* investigation involves the formation and growth of urinary stone crystals, most often, by using the gel methods. A focus is made on the results obtained on the formation and growth of Whewellite, Brushite, and Struvite crystals as these are among the dominant and well studied urinary stone crystals.

2. Urolithiasis

The occurrence of urolithiasis may take place due to anatomic features leading to urinary stasis, low urine volume, dietary factors (high oxalate is an example), urinary tract infections, systemic acidosis, medications, or uncommonly genetic factors such as cystinuria [1]. The most common contributory factors for the stone formation are: inadequate hydration (and subsequent low urine volume), hypercalciuria, hyperoxaluria, hyperuricosuria and hypocitraturia [12,13]. The formation of crystalline stone particles in the urinary system is a consequence of increased urinary supersaturation involving the process of nucleation, and further growth of the formed stone particles involves the processes of normal growth, oriented overgrowth (epitaxy) and aggregation [7,8]. It should also be considered that the crystallization starts at the nephron level and that the nephron parts are predisposed to the precipitation of different crystal types [8].

The major crystalline materials found in the urinary calculi are [8]: Calcium oxalate monohydrate (Whewellite), Calcium oxalate dihydrate (Weddellite), Hydroxyapatite, Carbonate-apatite, Calcium hydrogen phosphate dihydrate (Brushite), β-Tricalcium phosphate (Whitlockite), Octacalcium phosphate, Magnesium ammonium phosphate hexahydrate (Struvite), Magnesium hydrogen phosphate trihydrate (Newberyite), Anhydrous uric acid (in two structural forms), Uric acid dihydrate, Ammonium acid urate, Sodium acid urate monohydrate, Cystine, Xanthine, *etc*. The major types of urinary calculi found are [1]: Calcium stones occurring due to hyperparathyroidism, renal calcium leak, hyperoxaluria, hypomagnesemia, hypocitraturia, *etc.*; Uric acid stones forming due to pH < 5, high intake of purine foods (fish, meat, *etc.*), cancer, *etc.*; Struvite stones occurring due to gram negative-urease positive organisms (pseudomonas, proteus, klebsiella, *etc.*) that breakdown urea into ammonia; Cystine stones forming due to an intrinsic metabolic defect causing the failure of the renal tubules to reabsorb cystine, lysine, ornithine, arginine, *etc*. Also, several drugs like Atazanavir, Indinavir, Triamterene, Guaifenesin, (overuse of) Silicate and Sulfonamide have been known to promote stone formation [1].

Urolithiasis is considered to be a disorder having no temporal, geographical and demographic bounds; but is changing its pattern with time in the above aspects. The stone formation is essentially a physico-chemical phenomenon explicable in terms of the crystallization of sparingly soluble salts in an aqueous environment. The two main theoretical models proposed to understand the urinary stone formation are the so-called ‘free particle’ and ‘fixed particle’ models. The ‘free particle’ model suggests that the stone formation process is essentially extracellular (or intratubular); and the ‘fixed particle’ model suggests that the initiation of stones involves an intracellular interstitial or cell surface phenomenon [8,14].

The urinary stone crystals or crystalline aggregates have been found to range widely in their size, shape, color, composition and texture. The size ranges from that of a pinhead to that of the largest known weighing about 6.3 kg; the larger bladder stones weigh about 1 kg and the staghorn calculi (filling the calyces and pelvis of the kidney) weigh about 100 g [8]. Although it is more common to find formation of single stones than multiple ones, the formation at a time can range from one to few hundreds. The multiple stones found are faceted, often like rounded tetrahedral, small and round like a caviar, or sometimes as irregularly shaped with tiny spikes; the single stones found are round, ellipsoidal, highly irregular or like a hempseed, mulberry or jackstone [8]. The mechanical hardness is different for different urinary stones; some stones can be sectioned easily, some are crumble and others cannot be ground at all. Many stones have been found to have a well-defined nucleus surrounded by concentric bands of material; and others found to be conglomerates with no obvious nucleus [15].

2.1. Whewellite crystals

The three phases of calcium oxalate are: calcium oxalate monohydrate (COM, Whewellite), calcium oxalate dihydrate (COD, Weddellite) and calcium oxalate trihydrate (COT). COT is the most hydrated and kinetically favored, but, mostly transformed into COM which is preferred thermodynamically [5]. COD is less stable than COM thermodynamically and its formation may require the presence of pyrophosphate or citrate in the human urine [16, 17]. The occurrence of COM and COD in human urine depends on the molar ratio of calcium and oxalate [18]. The calcium oxalate stone formation possibly occurs due to: high oxalate / calcium ratio in urine (at a given level of supersaturation), low and high levels respectively of its crystal growth inhibitors and promoters, and a low urinary pH [18-24]. After crystal

nucleation, small as well as large molecular inhibitors possibly modify the growth and aggregation [25].

Calcium oxalate supersaturation can increase due to hypercalciuria, hyperoxaluria, hyperuricosuria, and low urine volume concentrations of citrate and magnesium; the citrate and magnesium ions have the ability to form soluble complexes with calcium and oxalate respectively [26, 27]. The calcium oxalate crystal formation can occur as a heterogeneous nucleation, promoted either by urine impurities or by macromolecules [28]. The uric acid and monosodium urate crystals formed in the urine also promote the calcium oxalate crystallization by the heterogeneous nucleation process. Even when the renal excretion of stone forming components is normal, a reduction in urine output to become half increases the urine saturation by more than two times [29].

The COT is highly unstable and is very rarely found whereas the COD and COM are commonly found together in urinary stones. COM crystal stones are smaller, more compact and are among the hardest stones having low fragility; these with no crystal interfaces are more resistant to shock wave lithotripsy [8]. The COM crystals formed *in vivo* in urine are brown in color whereas the *in vitro* formed ones are colorless and single or twinned or bunched prismatic crystals [30]. The COM crystal has a monoclinic lattice structure and becomes anhydrous at 200 ^{o}C [8]. Irusan [31] has reported that the COM crystals do not show any change in the crystal structure when grown in the presence of trace elements.

Iwata *et al.,* [32] have found that the architecture of small COM urinary calculi has three distinct zones. The first zone (core area) has randomly aggregated plate-like crystals, surrounded by the second zone (intermediate layer) showing prominent radial striations; this layer gradually shifts to the third zone (peripheral layer) where concentric laminations are prominent; and each lamination layer contains minute crystals having lost the radial arrangement. Studies made on COM crystals grown from gels exposed to various concentrations of nephrocalcin show that the habit, size and crystal structure are affected by this protein [8].

2.2. Brushite Crystals

Brushite (calcium hydrogen phosphate dihydrate) crystal is found to occur in calcified tissues, natural phosphate cavities and guano deposits; as incrustations on buried human and animal bones; and also found in biological systems as a component in human dental and urinary calculi [8]. Brushite can precipitate in urine with a pH below 6.9, a high calcium / magnesium ratio and a high calcium concentration; and other calcium phosphate phases predominate at higher pH values [8, 33, 34]. Also, through studies on crystalluria, it has been found that the calcium oxalate and calcium phosphate crystals predominate in urine in the pH range below 6.25 and above 6.5 respectively; Brushite is often formed in acidic urine and hydroxyapatite (HAP) is mostly formed in alkaline urine [8]. Morphologic analyses have shown the common presence of calcium phosphate as a central core of large calcium oxalate stones; and Brushite and octacalcium phosphate (OCP) have been found to be possible nucleating substrates for the formation of calcium oxalate stones [8].

The Brushite crystals formed *in vivo* in urine are colorless or of light yellow color. The gel grown (*in vitro*) crystals show different morphologies like dendrites, spherulites, platelets and needles [8, 35, 36]. It has also been found that the presence of dopants (Ni, Mg and Pb) increases the hardness of the Brushite crystal, which makes the stone removal difficult by way of crushing it inside the body [8]. Additives like solonum nigrum, inusaparadisiaca and phyllanthus nintri have been found to show inhibitory effect by reducing the mechanical hardness of the Brushite crystal; the hardness has been found to increase with the increase of inner reactant of the growth medium [8, 31]. The Brushite crystal has a monoclinic lattice structure and becomes anhydrous at 294 ^{o}C [8].

2.3. Struvite crystals

The Struvite crystals are formed by the reaction of magnesium solution with ammonium solutions of phosphates and they become white and pulverelent in a dry atmosphere; normally they are associated with infection presence and hence the name infection stones or infection induced urinary stones [8]. Clinically, the Struvite stones are classified as mushy stones, staghorn stones and bladder stones. People older than 60 years are normally found with Struvite stones; the female population is at higher risk (60–80% Struvite stone formers), which may be related to the increased urinary infections received by females [37, 38]. Several investigations have demonstrated that urease producing bacteria are required for Struvite stone formation in humans [37]. The normal urine contains in adequate concentrations for Struvite stone formation the components such as calcium, phosphate, magnesium and urea; also, urine with pH > 7.2 and ammonia presence are obligatory for the Struvite stone formation [8, 39].

The known principles the Struvite stone formation follow are: urinary supersaturation of the Struvite stone component ions induces the particle formation, then the microcrystals grow and aggregate to larger crystals and (if retained in the urinary tract) grow to full sized stones [37]. The upper urinary tract (the kidneys and ureters) is sterile, hence, any bacteria entering this environment are normally considered to be infection producing (pathogenic); the urease producing bacteria (on entering) produces the enzyme urease, which catalyzes the hydrolysis of urea to ammonia and

carbon dioxide; then the pH raises to > 8 and the Struvite crystals form spontaneously [40]. Rodman [41] has found that the Struvite crystals constitute only about 2-3% of the urinary stones but the clinical problems they produce are more than with any other stone type.

Stratful *et al.,* [42] have found that the increase of ammonium concentration increases the purity of the Struvite precipitate. It has also been found that the urine composition can influence the formation of stones consisting of Struvite and calcium phosphates [8]. The Struvite crystals formed *in vivo* in urine are colorless. The Struvite stones have been found to exhibit orthorhombic, coffin lid and also rectangular tubular shaped morphology [43]. Struvite crystallizes with orthorhombic lattice structure and becomes anhydrous at above 300 ^{o}C [8].

2.4. *In Vivo* studies

Several scientists have made analysis of trace elements like Mg, Cd, Fe, Zn and Pb present in urinary calculi and their influence on COM crystallization [44-48]; Mg and Zn have been found to inhibit calcium oxalate formation [44-47]. Several potent macromolecular peptides, glycoproteins, and RNA like materials, magnesium, pyrophosphate, citrate and naturally occurring polymers (small ion inhibitors) present in urine have been found to inhibit the crystal growth rate and aggregation of calcium oxalate [44, 45, 49, 50].

The proteins produced by the kidney cells (Tamm-Horsfall protein, uroprotein and nephrocalcin) and hippuric acid have been found to be inhibitors of nucleation, growth and aggregation of calcium oxalate crystals [51-53]. However, the Tamm-Horsfall protein has been found to promote calcium oxalate and calcium phosphate crystallization; this dual effect of some macromolecules on crystallization of urinary stone crystals may depend upon the ambient conditions [54]. Several non-physiological inhibitors (methylene blue, phytate, polyelectrolytes, chlorophylline and pentosan polysulfate have also been found; but, Gla (r-carboxyglutamc acid) has been found to promote the calcium oxalate crystallization [44, 55, 56].

Mg, Zn, citrate, fluoride, stannous ions and pyrophosphate have been found to inhibit calcium phosphate available in urine; some compounds inhibiting calcium phosphate crystal growth such as citrate and pyrophosphate are also effective on crystal aggregation [57-64]. The glycosaminoglycans particularly the heparin, hyaluronic acid and chondroitin sulfate have been found to be good inhibitors of calcium phosphate aggregation [59]. Origin and causes of Struvite stones have been studied by Garcia Raia *et al.,* [61]; whereas the diagnosis for an effective management of Struvite calculi to eliminate the stone burden and prevent the stone recurrences has been attempted by Gettman *et al.,* [62]. Citric acid has been used in varying concentrations in the dissolution treatment of Struvite renal calculi; and causes of phosphate stone formation have been reviewed thoroughly by Hesse and Heimbach [63].

A number of studies have been made to understand the effect of a variety of diets related to the stone formation in animals. A potassium citrate supplemented diet has shown a lower relative calcium oxalate saturation in healthy dogs [64]. Potential ability to increase the solubility of Struvite crystals has been found with high protein diets [8]. Feeding dry foods supplemented with urine acidifier (D-2-methionine or ammonium chloride) has been found to reduce the urinary pH and thereby the Struvite activity product in cats, which are clinically normal [65]. The effects of dietary Ca, Mg and P have also been studied on Struvite stone formation in the urinary tract of rats [8]. Dietary oxalate increase has not been found to change the universal stone formation and the type of stones formed [66].

Ingestion of mineral water with high Ca content has been found to increase the urinary Ca and decrease the urinary oxalate [8]. Prasad *et al.,* [67] have found that the Musa stem juice is able to reduce the formation and dissolve the preformed Struvite with traces of calcium oxalate. Salus *et al.,* [68] have studied the capability of phosphocitrate (when combined with an antibiotic) to inhibit the formation of infection stones in rats. It has also been found that the urine pH is more important than mineral intake in controlling the Struvite precipitation [8]. After incubation with urease, crystallization of Ca and Mg phosphates in synthetic urine and human urine has been found to be changed [8].

Wabner and Pak [69] have studied the urinary risk factors due to consumption of orange juice; the orange juice has been found to increase the inhibiton of Brushite formation and not to decrease the saturation of calcium oxalate in urine, when compared with potassium citrate. Sakhaee *et al.,* [70] have found that long term supplementaion of calcium citrate may not promote the crystallization of calcium salts in the urine; the effect of citrate on the crystallization of different calcium oxalates has also been studied [8]. Further, adhesion of urinary stone crystals to the apical surface of renal tubular cells blocked by specific anions like heparin; heparin sulfate, chondroitin sulfate C and hyaluronic acid have been studied [8]. Millan *et al.,* [71] have studied the effect of additives (EDTA, citrate and phytate) on the COM crystal growth kinetics. Moreover, it has been found that the inorganic pyrophosphate (present both in stone formers and non-stone formers) inhibits the growth of calcium phosphate and calcium oxalate [8].

The effect of different feed ratios on the urine pH value has been studied in cats; the urine pH is

significantly increased with Ca-carbonate or lactate, decreased with Ca-chloride, phosphoric acid and ammonium chloride, and no effect with calcium phosphate and ascorbic acid [8]. Mandel *et al.,* [72] have reported a strong trend for the conversion of stone disease (from calcium oxalate to calcium phosphate) and its influence on the progression and severity of the disease. Brichier *et al.,* [73] have thoroughly explained the basic conditions for the formation of urinary infection stones while Rahman *et al.,* [74] have recognized the relationship between the stone formation and urinary infections.

2.5. Medical therapy and treatment

Rosa *et al.,* [75] have reviewed several advances in the management of urinary stone disease; treating patients with urolithiasis requires a complete knowledge of metabolic evaluation and subgrouping of stone-forming patients, diagnostic procedures, effective treatment regime in acute stone colic, medical expulsive therapy and active stone removal. Urolithiasis is a dominant problem often correlated to diabetes, hypertension, high purine intakes, obesity and metabolic syndrome; hence, adequate patient information regarding dietary and drinking recommendations are more important in any pharmacological therapy [75]. Moreover, dietary, fluid intake and lifestyle factors are major research topics on prevention of the stone disease [76].

A non-contrast abdominal and pelvic CT scan is the most sensitive and reliable test for urolithiasis diagnosis, which also gives information related to concerns for infection or obstruction with resultant hydronephrosis [1,77-81]. When the CT scan is positive for urinary stones, doing a simultaneous KUB (kidney, ureter and bladder) can give more information useful to track properly the progress of the stone, its degree of calcification and its shape [82]. With medical expulsion therapy like tamsulosin, nifedipine and alfuzosin, stones of size < 5 mm have 90 % chance of passing on their own; but, urinary tract infection should be treated aggressively with antibiotic; moreover, acute management requires IV hydration, analgesia and antisemetic medications [1, 83, 84].

Urgent intervention is required in several cases: inadequate pain relief with oral analgesics, patient with a transplanted kidney and renal calculi, presence of renal calculi and pyelonephritis, *etc* [1]. Dissolution therapy may work for uric acid and cystine stones and does not work for calcium stones: sodium bicarbonate and allopurinol can be used to treat with uric acid stones; D-penicillamine, aggressive fluid intake and alkalinisation can be used to manage with cystine stones; and patients with recurrent stones can be recommended with thiazide diuretics [1]. Stones can be surgically managed in many ways: extracorporeal shockwave lithotripsy (ESWL, to break up stones anywhere in the urinary tract), ureteroscopy with laser lithotripsy (for stones in the lower ureter), percutaneous nephrolithotomy (for stones with size > 2 cm in the renal pelvis), *etc* [1]. As recurrence of renal calculi is more probable for patients with ongoing malignancy or metabolic problems, the patients have to stay hydrated; no medical therapy is successful without hydration [1].

3. *In vitro* Studies and Gel Methods for Crystal Growth

3.1. *In vitro Studies*

Formation and growth of crystalline particles in tubular fluid and in urine comprise two major physico-chemical aspects: (1) *thermodynamic* including supersaturation, which results in the nucleation of microcrystals, and (2) *kinetic* comprising rates of crystal nucleation, growth, aggregation and phase transformation, which in turn depends on the solution supersaturation. The rates of the four kinetic processes will determine phase, shape, size and number of crystals formed [7, 8].

The possibility of stone recurrence in a person has been reported to be 67-100% [8]. This indicates that mere stone removal cannot serve the purpose of curing the disease. So, a proper application of both the stone removal and drug therapy is required to control the urolithiasis. Surgical therapy leads to removal of stones already present whereas drug therapy aims at inhibiting the growth of existing stones and formation of new stones. Drugs are expected to be useful in preventing stone recurrence, avoiding renal colic, reducing the surgery requirement and correcting the extra renal manifestations of systemic diseases. Moreover, the cost of drug is considered to be less when compared to that of stone removal [8]. This makes it important to understand the stone formation mechanism as well as the identification of inhibitors and promoters of urinary calculi components (particularly the crystalline materials).

Enormous research efforts have been put (in the past) to understand the mechanism of stone formation, but without much success; the main reason for this is that the uroliths grow *in vivo* in complex biological fluids (influenced by various factors) and direct observation is difficult. However, as it is required to understand the situations that give rise to crystalline disease, it becomes necessary to investigate *in vitro* of the crystalline components present in the urinary stones.

The factors of solubility, nucleation and growth rate are same in both *in vivo* and *in vitro.* By knowing the growth and dissolution of the stone mineral phases and stones it is possible that the effect of various inhibitors present in urine can be studied. Moreover, the *in vitro* experiments artificially simulate the stone formation conditions to infer the general principles of calculogenesis.

Inhibitors are expected to increase the supersaturation required to initiate nucleation, inhibit secondary nucleations or reduce the growth rate and aggregation of crystal nuclei whereas promoters are expected to provide foreign surfaces for heterogeneous nucleation, growth and aggregation of crystals without altering supersaturation. At concentrations much lower than the supersaturation, the inhibiting and promoting compounds primarily adsorbing to surfaces of already formed crystals change the physico-chemical properties of crystal surfaces and thereby the rates of growth and aggregation.

The biological crystals as they develop in the human body and similar crystals grown in the laboratory in a homogeneous or heterogeneous environment of crystal growth are found to have very similar physical characteristics. So, the same physical principles can be utilized in the study of biological crystals and those crystals that are grown in the laboratory [8].

The association of urinary macromolecules with Ca-oxalate crystals induced *in vitro* in normal human and rat urine has been studied; and it has been understood that several urinary macromolecules including prothrombin-related proteins, osteo pontin and albumin are associated with Ca-oxalate crystals. Hounslow *et al.,* [85] have studied the Whewellite precipitation from supersaturated saline solutions as *in vitro* model of the formation of human kidney stones as well as a model of particle size enlargement during precipitation from solution. The changes in Ca-oxalate crystal morphology have been studied as a function of concentration and found that Whewellite is the predominant species at all concentrations [8]. Bretherton and Rodgers [86] have studied the crystallization of Ca-oxalate in minimally diluted (92%) urine. Effects of different diets including low calcium, high oxalate, vitamin C, high salt and lacto vegetarian on urinary risk factors for Ca-oxalate kidney stone formation have been investigated by Rodgers and Lewandowski [87].

Crystallization properties in urine has a serious problem that the place of formation of crystals that appear in urine is unknown. Crystalluria is considered to be the result of nucleation somewhere in the nephron and, at this level, the exact urine composition and supersaturation are not known; also are not known the types and concentrations of inhibitors and promoters of the crystallization process. Unfortunately, the *in vitro* experiments with urine samples in glass vessels do not reflect what happens *in vivo* as they do not have the cellular lining that is present always during physiologic crystallization in the nephron. Moreover, the impurities and debris added to the urine during its collection and preparation and the glass wall itself can result in crystallization that does not reflect the physiologic process [88].

The urine leaves the nephron and spends additional time in the rest of the urinary tract making the bladder urine with changed condition. Also, the composition of the urine is found to vary throughout the day making a 24 h collection reflecting the average of the day. Moreover, it makes a difference whether one uses whole urine (untreated), undiluted urine (treated to remove particles) or diluted urine [7, 8].

The major urinary stone crystals are found to have relatively low solubility and low thermal stability which lead to *in vitro* growth based on diffusion controlled and slow precipitation technique called gel growth. It has been shown that urinary stones grow in a gelatinous medium which is probably one of the reasons for the oriented striated growth of the crystal specifically encountered in the urinary calculi [8]. Gel acts as an inert medium during the growth of many crystalline compounds and it acts as an ideal medium in the study of the crystallization of biomolecules *in vitro* [8, 89]. Moreover, their viscous nature provides simulation of biological fluids in which biornolecules grow. Gel diffusion models have relevance because all stones crystallized in urine are coated with at least a monolayer of adsorbed polymer which forms a diffusion barrier and thus retards the rates of growth and dissolution [90].

This attracted several research workers to grow in gel media the major crystalline components of urinary stones, which includes the growth studies in the presence of substances acting as inhibitors or promoters. It has been found that drugs like gentamycin are suspected to induce Whewellite nucleation [8]. Pyridoxin, allopurinol and citrate have been found to inhibit the formation of oxalate crystals; Allopurinol inhibits the formation in the presence of uric acid, otherwise, it induces the formation [54, 91, 92].

Now the question is whether the *in vitro* studies of the above type give the required result with the drugs (normally introduced into the body orally) which undergo metabolic changes before reaching the kidney or urinary tract [93]. The answer is in the positive side as understood by the studies made in the present author's laboratory with gentamycin and allopurinol with Whewellite crystal [94]. Similar results were obtained in the present author's laboratory [94] in comparison with the results reported in the medical literature [54, 91, 92]. Still, in principle, no authentic statement can be made for drugs undergoing metabolic changes whether the drug has a side effect of promoting the formation of urinary stone crystals in the urinary tract or not. However, results of this type of *in vitro* studies may probably help to carry out further study in treatment of urinary calculi. Moreover, *in vitro* studies made by growing the crystals in gel media are superior to those made with urine because the conditions can he controlled to a higher level of accuracy in gel media.

Girija and her co-workers [8, 30] have grown Whewellite single crystals by using the gel method and investigated the influence of the presence of trace elements such as Mg, Cd, Zn and Pb in the urinary calculi. Deepa *et al.*, [95] have made the surface topographical studies of Ca-oxalate crystals grown in silica gel. The effects of individual glycoaminoglycans (GAG) species on the Whewellite crystal growth have been studied by observing the crystal morphology and found that the CAGs affect the morphology of Whewellite crystals differently depending on the species [8].

Several researchers have reported [8, 36, 96, 97] the *in vitro* formation of Brushite crystals in gel media; and have found that the optimum gel density for the crystal growth is 1.03 g/cc. Deepa [98] has found that addition of citric or tartaric acid alters the *in vitro crystal* growth significantly by reducing the growth rate and changing the surface morphology. Ohta and Tsutsumi [96] have investigated the growth morphology and surface structure of Brushite crystals at fairly high growth rates in silica gel.

Struvite crystals have also been grown in gel media by several researchers [9, 99-101]. Sivakumar [10] has studied the role of ionic species (Mg^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, citrate ions and $P_2O_7^{4-}$) on the crystallization of dicalcium and magnesium phosphates. Irusan [31] has studied the effect of impurities like ammonium, lead, barium and cadmium on the morphology of Struvite crystal.

3.2. *Gel Methods for Crystal Growth*

Valuable information on various gel methods can be obtained from a book by Henisch [102] and some other articles [103-105]. A gel is a loosely interlinked polymer and gelling process can be done in several ways: by cooling of a sol, by chemical reaction, by the addition of precipitating agents or incompatible solvents, by the action of two reagents in concentrated solution, *etc*. Depending on the nature of the material, its temperature and history the gelling process takes an amount of time varying widely from minutes to many days. The mechanical properties of a fully developed gel can vary widely depending on the density and precise condition during gelling. The gel medium remains chemically inert and prevents turbulence which provides a three-dimensional structure permitting the reagents to diffuse at a desirable controlled rate. The gel method has the advantage of being observed practically in all stages of crystal growth. The viscous nature of gel provides simulation of biological fluids in which the crystals of biominerals grow, and the gel acts as an ideal medium in the *in vitro* crystallization of biominerals [89].

Silica gel (prepared from sodium metasilicate solution) is the best and most versatile growth medium for the *in vitro* formation and growth of urinary stone crystals (particularly Whewellite, Brushite and Struvite) [8]. Silica gel can be prepared by mixing aqueous solutions of sodium metasilicate (of density 1.03-1.08 g/cc) and acid (1-4 N of mineral or organic). When sodium metasilicate goes into solution, monosilicic acid is considered to be produced. It is accepted that monosilicic acid can polymerize with the liberation of water which can happen again and again until a three dimensional network of Si-O links is established, as in silica. This water accumulates on top of the gel surface as the polymerization process continues, a phenomenon known as syneresis. For silica gels, estimates yield effective pore diameters of the order of 50 - 160 Å. The gel acts as a 'three-dimensional crucible' supporting the crystal and yields to its growth without exerting major forces upon it. Moreover, the basic gel structure is not expected to affect the crystal growth characteristics like growth rates and ultimate crystal size.

Various dense silica gels can be obtained (and used for crystal growth) from a stock solution of sodium metasilicate, prepared by adding 500 ml of water (distilled or demineralized) to 244 g of $Na_2SiO_3.9H_2O$. In order to avoid absorption of carbon dioxide the stock solution should be kept from contact with the atmosphere. The reaction method (single diffusion as well as double diffusion) is the most important method used for the *in vitro* studies on the formation and growth of urinary stone crystals (particularly Whewellite, Brushite and Struvite) [8].

In this method, two soluble (in the solvent - usually water) reactants diffuse through the gel medium where they react and form an insoluble or relatively less soluble (in the solvent) crystalline product. The crystal formation and growth can be done by the test tube technique (in which one of the reactants is incorporated inside the gel and the other reactant is diffused into it) or by the U-tube technique (in which the two reactants are allowed to react by diffusion into an essentially inactive gel). Crystals of Whewellite, Brushite and Struvite can be grown by adopting the methods similar to that used by the present author and his co-workers [8, 93, 94, 100, 106-110].

3.2.1 *Growth of Whewellite Crystals*

For growing Whewellite crystals [8, 94, 106-109], the U-tube technique (with tube limbs of diameter 2.5 cm) is considered and the desired pH (6.0–6.5) of the gel solution is obtained by adding 1.5 M glacial acetic acid to the sodium metasilicate solution of density 1.03 g/cc. After gelation in about 12-24 h and ageing for about 24-36 h, 12 ml of 1 M calcium chloride solution mixed with 8 ml of 1 M magnesium acetate solution (a total of 20 ml) is added to one limb of the U - tube. 20 ml of 1 M oxalic acid solution is added to the other limb of the U-tube. The U-tube is kept at a temperature of 36.9 °C (normal human body temperature) in a constant temperature bath.

3.2.2. *Growth of Brushite Crystals*

For growing Brushite crystals [8,93,100,110], the test tube technique (with straight tube of diameter 2.5 cm and volume 50 ml) is considered and the desired pH (5.0 - 6.5) of the gel solution is obtained by adding 2.0 - 2.5 M orthophosphoric acid. After gelation in about 12 h and gel ageing of 24 h, 10 – 15 ml of 1.5 M calcium chloride and 5 ml of 1.5 M calcium acetate were carefully poured over the gel. The straight tube is kept in a constant temperature bath at the normal human body temperature of 36.9 °C.

3.2.3. *Growth of Struvite Crystals*

For growing Struvite crystals [8,93,100], the test tube technique (with straight tube of diameter 2.5 cm and volume 50 ml) is considered and the desired pH (6.0 - 7.0) of the gel solution is obtained by using glacial acetic acid. The gel was set after incorporating ammonium dihydrogen phosphate (of 1.5 M concentration) within the gel. After gelation in about 12 h and gel ageing of 24 h, magnesium acetate solution (of 1.5 M concentration) is gently poured over the gel. The straight tube is kept at the normal human body temperature of 36.9 °C in a constant temperature bath.

3.2.4. *General Observations and Total Product Mass Determination*

Observations can be made (during the formation and growth of urinary stone crystals in the gel media) on the size, shape, transparency and approximate number of crystals obtained to derive conclusions regarding the promotely / inhibitory effect of drugs, juices and / or other addents / impurities incorporated (along with the precursors used for crystallization). Also, the harvested crystals (including tiny ones) can be weighed (to an accuracy of ± 0.1 mg using a sensitive balance) to obtain the mass of the total product got after crystallization. Although this measurement is inaccurate [as very tiny crystals (powder like) cannot be included for the measurement] this is done to understand qualitatively the effect (inhibition / promotion) of drugs, juices and / or other addents / impurities on the formation and growth of urinary stone crystals [8, 93, 110].

Moreover, the crystals grown can be characterized structurally, chemically, thermally and mechanically by subjecting them to density, X-ray diffraction, spectroscopic (FT-IR, EDAX, *etc.*), thermal (TG, DTA, *etc.*) and microhardness measurements. It has been found [111] that the ionic nature of the urinary stone crystals can be used to study the nucleation and ensuing crystal growth and thus inhibiting their formation. The mobility of ions in urinary stone crystals is more dependent on the field (thermal and electric) in the human body temperature range which is expected to have certain biological significance [112]. This indicates that study of the thermal parameters becomes imperative in finding a potential urinary stone crystal inhibitor. Debye temperature can be estimated from the X-ray diffraction data; the increase in Debye temperature due to inhibitor (impurity) addition is expected to result in poor chance of formation of the urinary stone crystals [113].

4. Effects of Drugs and Juices

In this Section is presented a review of the *in vitro* studies carried out to understand the effect of drugs and juices on the formation and growth of Whewellite, Brushite and Struvite crystals; the focus is made on the work of the present author and his co-workers.

Irusan [31] has reported the *in vitro* studies on the effect of changing the growth parameters and the effect of adding impurities (juices of medicinal plants like phyllanthus niruri, octimum sanctum and solanum nigrum) on the growth of some urinary stone crystals.

The present author and his co-workers [8,93,94,106,110] have planned and executed a systematic investigation (can be considered as an attempt, more of qualitative than quantitative) to understand the effect of three antidiabetic drugs, *viz.* Glyciphage (metformin hydrochloride), Daonil (glibenclamide) and Semiglynase (a lower dose of glynase, glippizide) on the formation of Whewellite, Brushite and Struvite crystals; and also investigated the effect of juices (extracted with seeds removed) of tomato, lime and cucumber (advised for the diabetic patients to take frequently) in balancing the effects caused by these antidiabetic drugs. The crystals were grown in silica gels by using the double diffusion (for Whewellite) and single diffusion (for Brushite and Struvite) methods.

The antidiabetic drugs considered were added separately with four (10, 20, 30 and 40 mg in the case of Brushite and Struvite) to five (10, 20, 30, 40 and 50 mg in the case of Whewellite) concentrations in each case; the juices considered were added separately with four dosages (0.125, 0.250, 0.375 and 0.500 ml) in each case; the juice (with a single dosage of 0.5 ml) and drug (with a single dosage of 50 mg for Whewellite and two dosages of 20 and 40 mg for Brushite and Struvite) were added together to understand the balancing effect of juice with the drug. The drug or / and juice were added along with oxalic acid in the case of Whewellite, calcium chloride and calcium acetate in the case of Brushite and magnesium acetate in the case of Struvte.

The physico-chemical analyses through X-ray diffraction, density, thermogravimetric and FT-IR spectral measurements have shown that the lattice structures of the urinary stone crystals considered are not distorted by the addition of drug or / and juice used. In order to derive conclusions regarding the promotery / inhibitory and balancing effects of the drug or / and juice incorporated, observations were made on the size, shape, transparency and approximate number of crystals

obtained; the total product mass determination was also carried out. The total product mass determined and crystals formed indicate that all the three drugs considered (Glyciphage, Daonil and Semiglynase) have promotery effect (except Semiglynase in the case of Struvite) on the growth of all the three urinary stone crystals considered (Whewellite, Brushite and Struvite). All the three juices considered (of tomato, lime and cucumber) are found to be inhibiting (or balancing) the promotery effect of the drugs considered.

Metformin is normally excreted unchanged in the urine and it neither undergoes hepatic metabolism nor biliaiy excretion. So, it may be mentioned that Glyciphage has a side effect of promoting the formation of urinary stone crystals in the urinary tract; but, no authentic statement can be made in this direction with the other two drugs (Daonil and Semiglynase). However, the results reported by the present author and his co-workers may probably help to carry out further study in the treatment of urinary calculi.

In addition, the present author along with his co-workers [100, 107-109] have made investigations to understand the effect of some commonly consumed allopathic drugs (*i.e.* to understand the side effect of these drugs), *viz.* Dygine, Gelucil MPS, Crocin, Paracitamol, Disprin-plus, Disprin, Aspro and Novalgin on the formation of Whewellite, Brushite and Struvite crystals. Dygine and Gelucil MPS are most often used for stomach disorder; Crocin, Paracetamol and Disprin-plus are most often used for killing fever; and Disprin, Aspro and Novalgin are most often used for killing head ache. The crystals were grown without and with adding these drugs (at four different dosages, viz. 10, 20, 30 and 40 mg in each case) in silica gels by using the double diffusion (for the Whewellite) and single diffusion (for the Brushite and Struvite) methods. It has been established that all the eight allopathic drugs considered are strongly promoting the formation (as a side effect) of the Whewellite, Brushite and Struvite crystals.

Freeda and her co-workers [114, 115] have studied the effect of some siddha medicines (most often used for the treatment of diabetes) like mathumaga chooranam, thribala chooranam and neem leaf juice on the growth of Brushite and Struvite crystals by using the single diffusion gel growth method. They have found that these siddha medicines are highly capable of inhibiting the growth of these crystals.

Recently, Anushya and Freeda [116, 117] have investigated the influence of green tea (in various amounts) and some fruit extracts prepared from natural foods on the formation of Brushite crystal by using the single diffusion gel growth technique. The natural foods they have used are *solanum lycopersicum* (tomato), *daucas carota* (carrot) and *vitis vinifera* (grape), which are used (in the Aurvedic system of medicine) for the treatment of various human ailments like kidney stone, diabetes, urinary tract infection and immune system. They have found that these green tea and fruit extracts have good inhibitory effect on the growth of Brushite crystal.

Very recently, Anushya and Freeda [113] have investigated the effect of some allopathic and siddha medicines such as Cetapin XR, Glucored forte, Glycomet, Aegle marmelos (leaf extract), Andrographis paniculata (leaf extract) and Cassia auriculata (flower extract) on the growth of Brushite and Struvite crystals and found that these medicines significantly inhibit the formation of these crystals. The crystals were grown by using the single diffusion gel growth method and 50 mg medicine was added in the supernatant soluton taken over the gel.

5. CONCLUSION AND FUTURE SCOPE

The formation and growth of urinary stone crystals (in fluids in the urinary system) occur due to physico-chemical aspects like supersaturation, crystal nucleation, growth, aggregation and phase transformation. It has been found that we can use the same physical principles while studying the biological crystals (grown *in vivo*) as well as those grown (*in vitro*) in laboratories. Moreover, it has been found that most of the commonly consumed allopathic drugs (or their metabolites) used by patients may promote the stone formation (may be as a side effect).

The information presented in this article is only a limited part of this broad research field, which requires much more to be investigated in the future. Similar work is to be done to understand about all the significant crystalline components of urinary calculi; and investigations have to be made to understand the promotery / inhibitory effect of other widely used drugs, medicinal herbs, soft drinks and hard drinks. Trials with known inhibitors can also be made to understand the dissolution kinetics of already grown crystals; this can possibly be used to cure natural stones *in vivo*, instead of going for painful surgery and all. Often urinary stones contain more than a particular crystal type, rather a mixture of several types with one or two as dominant; so, studies should be extended to mixed stones crystals also.

This type of research will be highly useful to the society in preparing new drugs for treatment to various diseases; and also to understand the role of every component (of the drug) in forming the stone crystals. Moreover, authentic conclusions can be made in a better way by going deep into the pharmacology of drugs and the body metabolism. Further progress can also be achieved by linking this field in a considerable level with fields like Biochemistry, Biophysics, Mineralogy, Nephrology, Pathology and Urology.

ACKNOWLEDGEMENT

The support by the Council of Scientific and Industrial Research, New Delhi, India under the Emeritus Scientist Scheme (CSIR-ES Scheme No.: 21(1083)/19/EMR-II) is hereby gratefully acknowledged.

Funding: Preparation of this article received no specific grant from any funding agency.

Conflict of Interest

The author declares that this article was prepared in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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