URINARY FACTORS AFFECTING RENAL STONE DISEASE

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A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

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CERTIFICATE OF ORIGINALITY

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POON Ngork Wah (Name of Student)

Abstract

This study investigates the physicochemical aspects of calcium oxalate (CaOx) crystallization. Two major classes of crystallization modifiers were studied – urinary glycoproteins (GPs) and urinary glycosaminoglycans (GAGs) by the mixed suspension mixed product removal (MSMPR) system – a continuous crystallization system. Total urinary GPs were shown to promote CaOx crystallization. Although whole urinary GAGs showed no significant differences on CaOx crystallization, individual GAGs showed that chondroitin sulphate and heparan sulphate promoted the CaOx crystallization while hyaluronan (HA) promoted the formation of smaller CaOx crystals but showed no significance in the crystal density of CaOx formed in the urine.

Since HA is reported as a promoter of CaOx crystallization which is a distant form of other GAGs species, we studied both total urinary GAGs and HA excretion in stone-formers (SF), post-treated SF and compared with normal individuals. Active-SF and post-treated SF groups had lower total GAGs excretion but increased proportion of HA than that of the normal controls indicating that urinary GAGs and HA are probably protective/risk factors for the occurrence and recurrence of renal stone disease.

CD44 is known as a HA receptor and CD44 is found on various cell surfaces including epithelial cells and shown to bind with HA when released by cells in several studies due to cell injury induced by inflammatory response. Therefore, HA excretion and CD44 expression in renal cells as an inflammatory response and HA expression on CaOx induced cell injury were also studied. This study suggested the possible events

during subclinical CaOx renal stone disease. HA was secreted during CaOx induced cell injury and the injured cells will also secrete IL-1β as the inflammatory cytokine. This can eventually lead to further HA secretion as well as CD44 expression that can interact with the HA being secreted for CaOx crystal attachment and retention for stone formation during the healing of the injured renal cells.

Melamine renal stone disease incident in Chinese infants in late 2007 lead to the concern on how melamine affected renal stone disease. Unlike CaOx crystallization, the physicochemical nature of melamine in urine and its handling were largely unknown at that time. Therefore, the well-established MSMPR system on CaOx crystallization was applied for melamine crystallization studies.

The physiochemical properties of melamine crystallization including pH and ionic strength were studied. Melamine was found to crystallize in normal acidic urinary pH (pH 6.0) so that normal urine can provide an optimal condition for melamine crystallization. Besides, it was also found that melamine can interact with other lithogenic salts including calcium, oxalate and uric acid. Presence of melamine significantly enhanced the precipitation of calcium oxalate while the presence of uric acid significantly reduced melamine crystallization.

Urinary tract infection (UTI) is common in children. As melamine stones occurred in infants and children, the main pathogen of UTI – *E. coli* on melamine crystallization was studied. *E. coli* promotes melamine crystallization and thus

appropriate measure such as treating UTI prior to stone removal can reduce the severity of melamine stone disease and the complications of UTI.

Currently used therapeutic agents on CaOx stone disease were also prescribed on melamine stone subjects. However, the effectiveness of these drugs on melamine crystallization was largely unknown. Therefore, the effects of currently prescribed therapeutic agents including potassium citrate and sodium bicarbonate on melamine crystallization were investigated. Both citrate and bicarbonate did significantly reduce melamine crystallization so that they were effective against melamine crystallization and melamine stone formation.

Traditional Chinese medicines (TCM) have been used for treating renal stones due to their anti-lithogenic activities. Previous study found that a Chinese herb Shi Wei (Folium Pyrrosiae) has a potential to inhibit urinary crystallization by reducing the urinary specific gravity and enhancing the urinary magnesium. This study showed that the human urine after Shi Wei supplementation had significant acute inhibitory effect on melamine crystallization but the effect was diminished or even levelled off after 1 week supplementation. Thus, Shi Wei may also be a suitable therapeutic agent for the quick control of melamine stones in infants by inhibition of further melamine crystals formation.

Publication arising from the thesis

Papers

1. Yuen, J.W.M., Gohel, M.D.I., **Poon, N.W.**, Shum, D.K.Y., Tam, P.C., Au, D.W.T.

   The initial and subsequent inflammatory events during calcium oxalate lithiasis.

   Clinica Climica Acta, 2010. **411:** 1018-26.

2. **Poon, N.W.,** Gohel, M.D.I.

   Urinary glycosaminoglycans and glycoproteins in a calcium oxalate crystallization system.

   Carbohydrate Research, 2012. **347:** 64-8.

3. **Poon, N.W.,** Shum, D.K.Y., Tam, P.C., Gohel, M.D.I.

   Hyaluronan is an accidental risk factor for recurrent stone formers.

   Urological Research, (Under review)

4. **Poon, N.W.,** Gohel, M.D.I., Lau, C., Hon, E.K.L., Leung, P.C., Ng, C.F.

   Melamine crystallization: the physiochemical properties, interactions with others lithogenic salts and response to therapeutic agents.

   Journal of Urology, 2012. **187:** 1483-90.

Abstracts for presentation

1. **Poon, N.W.,** Shum, D.K.Y., Tam, P.C., Gohel, M.D.I.

   Reduced excretion of glycosaminoglycans in active stone-formers and stone-formers post-ESWL.

   2nd International Urolithasis Research Symposium, 2008 (Indianapolis, Indiana, USA) (Poster)

2. Ng, C.F., **Poon, N.W.,** Lo, A, Gohel, M.D.I.

   The *in-vitro* crystallization of melamine and its effect on calcium oxalate crystallization.

   27th World Congress of Endourology and Shock-wave lithotripsy, 2009 (Munich, Germany) (Poster)

3. Gohel, M.D.I., **Poon, N.W.**, Ng, C.F.

   Environment: Learning from melamine.

   6th EULIS Symposium, 2009 (Lake Como, Italy) (Plenary – Oral)

4. Ng, C.F., **Poon, N.W.,** Hon, E., Gohel, M.D.I.

   The effects of physiochemical changes in urine on melamine crystallization.

   Health Research Symposium, 2010 (Hong Kong) (Poster)

5. Ng, C.F., **Poon, N.W.,** Hon, E., Gohel, M.D.I.

   The effects of therapeutic agents in the treatment of urinary stones on melamine crystallization.

   Health Research Symposium, 2010 (Hong Kong) (Poster)

6. Ng, C.F., **Poon, N.W.,** Hon, E., Gohel, M.D.I.

   The effects of Shi Wei (Folium Pyrrosiae) on melamine crystallization.

   Health Research Symposium, 2010 (Hong Kong) (Poster)

7. Gohel, M.D.I., **Poon, N.W.**, Hon, E., Ng, C.F.

   The effects of uric acid and calcium phosphate on urinary melamine crystallization.

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List of abbreviations

ANOVA	One-way analysis of variance
AU	Artificial urine
Bo	Nucleation rate (numbers/minute/mL)
BSC-1	Nontransformed African green monkey cell line
CaCl	Calcium chloride
CaOx	Calcium oxalate
CaP	Calcium phosphate
CE	Cellulose Ester
CFU	Colony forming units
CIRF	Clinically insignificant residual fragments
COM	CaOx monohydrate
CPC	Cetylpyridinium chloride
CS	Chondroitin sulphate
DMEM	Dulbecco's modified eagle's medium
DS	Dermatan sulphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMU	Early morning urine
ESWL	Extracorporeal shock wave lithotripsy
EtOH	Sodium acetate-saturated ethanol
FBS	Fetal bovine serum

FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FP	Formation product
G	Growth rate (µm/minute)
GAGs	Glycosaminoglycans
GPs	Glycoproteins
HA	Hyaluronan
HK-2	human kidney proximal epithelial cell line
HPLC	High performance liquid chromatography
HPV-16	Human papilloma virus 16
HS	Heparan sulphates
IL	Interleukin
ITI	Inter-α-trypsin inhibitor
KS	Keratan sulphate
MCP-1	Monocyte Chemoattractant Protein-1
MDCK	Madin-Darby canine kidney
MgOx	Magnesium oxalate
MH-S	Murine alveolar macrophage cell line
MIP	Macrophage Inflammatory Protein
ML	Metastable limit
MSMPR	Mixed suspension, mixed product removal
M_T	Suspension density (mmol/L or mM)
M.W.	Molecular weight

NaOx	Sodium oxalate
OPN	Osteopontin
PBS	Phosphate buffered saline
PCNL	Percutaneous nephrolithotomy
PMA	Phorbol 12-myristate 13-acetate
PT	Prothrombin
PTC	Proximal tubular cell
SF	Stone-formers
SSPs	Semi-synthetic polysaccharides
TCM	Traditional Chinese medicines
THP	Tamm-Horsfall protein
TNF	Tumor necrosis factor
UA	Uric acid
UMMs	Urinary macromolecules
UPMs	Urinary polyanionic macromolecules
UPTF-1	Urinary prothrombin fragment-1
UTI	Urinary tract infection
WHO	World Health Organization

Chapter 1: Introduction

1.1 Physicochemical basis of stone formation

Urinary stone disease is one of mankind's oldest diseases since crystallization in the urinary tract happens opportunistically and quite commonly [1, 2]. The formation of renal stones is a consequence of increased urinary supersaturation with subsequent formation of crystalline particles [3]. Hence, the urine is supersaturated with slightly soluble materials that can crystallize. The most common one is calcium oxalate (CaOx) [4, 5]. Normally, most of the solid particles crystallizing within the urinary tract will be excreted freely. However, when solid particles are retained within the kidney and the urinary tract, they can develop to full-size stones [6].

1.1.1 Crystalluria

Crystalluria is the presence of urinary crystals. It is well known that stone formation is a biological process that involves a physiochemical process, that is crystallization. However, crystalluria itself does not indicate renal stone disease because the crystals formed may be small enough to be excreted in the urine freely so that no renal stone is formed such as those in normal individuals [3].

1.1.2 Particle formation in the kidney

The formation of crystalline particles in tubular fluid and urine consists of two major physiochemical aspects: a thermodynamic one including supersaturation that results in nucleation of microcrystals, and a kinetic one including rates of crystal nucleation, growth, aggregation, and phase transformation [3], which in turn depends on solution supersaturation. The rates of the four kinetic processes will determine phase, shape, size, and number of the crystals formed.

1.1.3 Supersaturation

Supersaturation represents the excess of free energy, that is, the thermodynamic driving force, which is needed for crystal formation [7]. Supersaturation is undeniably essential for kidney stone formation due to the fact that formation of crystalline particles must obviously start from supersaturation. Actually, stone formers tend to excrete urines that are more supersaturated than those of non-stone formers [3].

1.1.4 Crystal nucleation

A supersaturated solution will try to reach a stable equilibrium by depositing excess material in solution as solid phase in order to shed its free energy [3]. Crystal nucleation is the initial kinetic step that allows for transformation from liquid into a solid phase in a supersaturated solution [8]. This process begins with the stone salts in solution aggregated as loose clusters that may increase in size by addition of new components or clusters [8]. Secondary nucleation then occurs when new crystals nucleate on pre-existing crystal surface of their own species [3]. However, many foreign surfaces such as cell debris, epithelial membranes, hyaline casts and various crystal species will constantly be present in tubular fluid and also in human urine. These impurities allow heterogeneous nucleation to occur for which crystals nucleate on such foreign surfaces [3].

1.1.5 Crystal growth

Crystal growth occurs when a crystal nucleus has achieved its critical size and the urine is supersaturated, overall free energy is decreased by adding new crystal components to the nucleus. The driving force for crystal growth is the net difference between the energy needed for formation of new crystal lattice bonds and the energy consumed for the desolvation both of the crystalline components in solution that are to be incorporated into crystals and of the crystal lattice itself [3].

1.1.6 Crystal aggregation

Aggregation or agglomeration is the process by which crystals in solution attach together to form a larger particle. The formation of a new, larger aggregate by adhesion of several particles is energetically favored and it is a natural process [3]. Aggregation occurs at all states of saturation although the rate may be influenced by the saturation state of a solution [3]. Besides, aggregation is a very fast process so larger particles can be formed within seconds [3].

1.2 Nature of stone matrix

1.2.1 Crystalline material

Renal stones themselves consist of about 98% crystalline material [1, 2]. The crystalline components of stones consist mainly of calcium salts including calcium oxalate (CaOx) and calcium phosphate (CaP) [9]. Nevertheless, most stones are mixtures of several different crystal types with one or two that are predominant [3]. The most common crystalline component of human renal stones is CaOx due to its low solubility and the high urine concentrations of calcium and oxalate normally excreted so that it is supersaturated [5].

1.2.2 Stone matrix

Renal stones do not consist of crystals alone as biomineralization requires an organic material called “matrix” for which the mineral has to be deposited on [8]. Therefore, stone matrix can be considered as the ‘framework’ of the stone. The stone matrix contains the organic material which may be derived from substances normally present in urine or may be produced by epithelial cells in the urinary tract subsequent to the trauma induced by an enlarging stone [3].

1.2.3 Modifiers of crystallization

Crystallization processes are greatly dependent on solution composition in tubular fluid as well as urine. Some urinary constituents may affect solution supersaturation because of their action as chelators. For example, citrate and magnesium reduce free ion activity and relative supersaturation of CaOx by forming soluble complexes with calcium and oxalate, respectively [3]. This efficiently diminishes not only the thermodynamic driving force, but also the kinetic rates of crystallization. Generally, there are two types of modifiers known as inhibitors and promoters [3]. Inhibitors are any compounds that impede crystallization processes while promoters are substances that enhance urinary supersaturation.

1.2.4 Particle retention in the kidney

The development of renal stones is mainly due to the retention of particles within renal tubules. Only a retained particle can have enough time to grow continuously and finally become a stone. Theoretically, retention is the particle movement down the urinary stream that is slower than that of the surrounding urine [6, 10]. In principle, retention can occur due to either particles becoming too large to pass freely through the tubules, or they adhere to tubular cell surfaces. Thus, a fixed particle mechanism is invoked for which CaOx crystals adhere to the glycocalyx of renal tubular cell membranes, as the glycocalyx is sticky [6]. In fact, membrane fragments of renal epithelial cells promote crystallization from supersaturated CaOx solutions [11].

The adherence of CaOx crystals increases with the number of crystals and shows concentration-dependent saturation [12]. The crystals bind irreversibly to cell surfaces and are subsequently endocytosed [13-15]. It was suggested that after erosion of the epithelium, further crystal growth occurred from the plaque into the renal pelvis [10]. The injury is a prerequisite for crystal adhesion as transitional epithelium is normally not sticky [16, 17]. Other studies showed that an intact and functional epithelium formed by renal tubular cells with characteristics of the late nephron is non-adherent to crystals [18, 19]. However, the non-adherent property of the renal tubular cells is lost after epithelial injury and during repair [19]. Therefore, the formation and subsequent retention of large particles in renal tubules is to be expected after cell injury. It may be the important first step in the formation of CaOx renal stones. The retained large particle becomes attached to the tubular cells by the cell-crystal interactions, or by damaging the tubular cell lining and reaching the basement membrane [20]. The retained particle finally grows into a full-size stone by accumulating further inorganic and organic material.

1.3 Urolithiasis (Renal stone disease)

Urolithiasis is the presence of renal stone and it is a common disease that is associated with high morbidity and recurrence rate due to the repeated stone episodes which leads to the economic burden [21] of urolithiasis. A retrospective study on idiopathic calcium urolithiasis showed recurrence rates of 14% at 1 year, 35% at 5 years, and 52% at 10 years [22]. Urolithiasis accounts for approximately 200,000 hospitalization per year in United States [22] and around 13,000 hospitalization per year in Hong Kong (Figure 1.1) over the past 13 years [23].

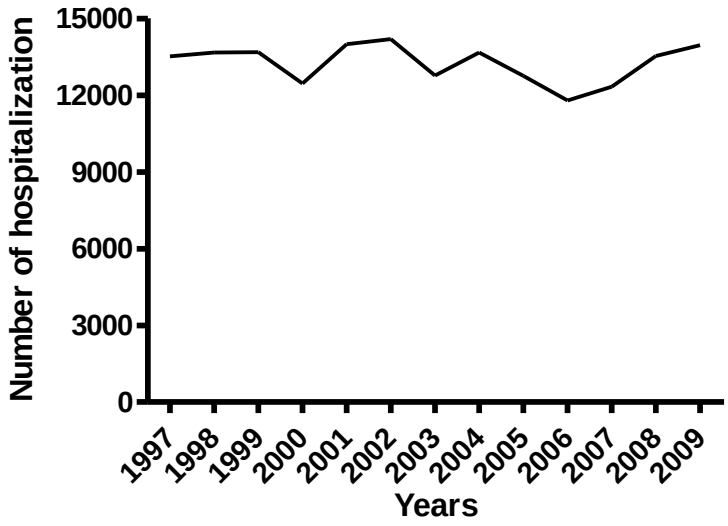


Figure 1.1. Number of hospitalization over the past 13 years in Hong Kong due to urolithiasis (Source: Hospital Authority Statistical Reports 1998-2010)

1.3.1 Clinical presentation of urolithiasis

The presence of a urinary stone (urolithiasis) causes an excruciating unilateral flank or lower abdominal pain of sudden onset (renal colic) that cannot be relieved by postural changes or non-narcotic medications. Gastrointestinal symptoms are usually absent unless the celiac plexus is being stimulated that result in nausea and vomiting [24].

1.3.2 Diagnosis of urolithiasis

Once the patients with abdominal pain is attended by the doctor, the history of past and family history of urolithiasis should be asked. On the other hand, physical examination should be done to rule out non-urologic disease. Urinalysis should be performed in all patients with suspected calculi since that may help to identify the stone type by some important findings such as the urine pH and the presence of crystals. Then diagnostic imaging should be done on patients with suspected renal colic to detect the presence of stone (Figure 1.2) as well as to confirm the size and location of urinary tract calculi [24].

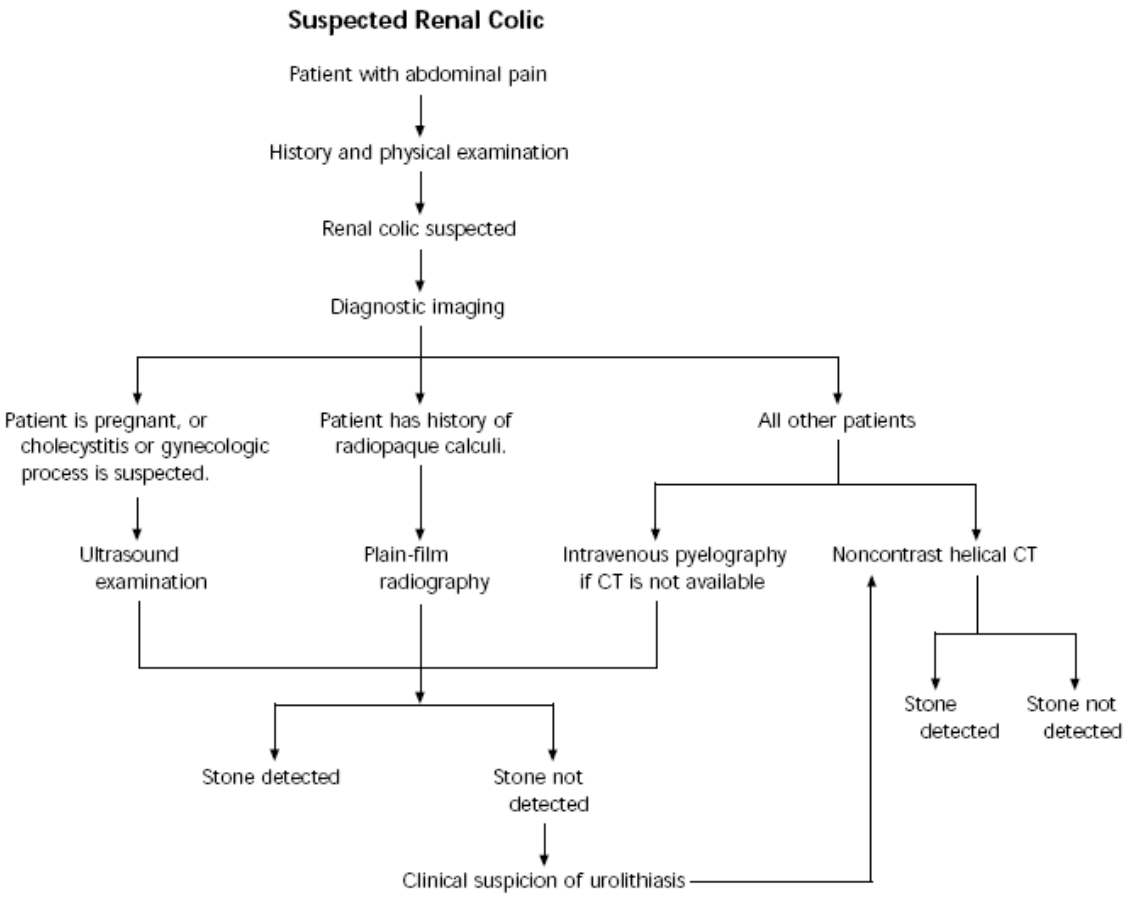


Figure 1.2. Diagnostic approach to urolithiasis. (Portis & Sundaram 2001)

1.3.3 Management of patients with urolithiasis

Generally, the initial step is to identify patients who are in an emergency situation such as urosepsis, anuria and renal failure (Figure 1.3). They should have urgent urologic consultation [24]. For example, patients with urosepsis in conjunction with an obstructing stone should establish an adequate drainage of the urinary system by means of percutaneous nephrostomy or retrograde ureteral stent insertion.

For other patients who are unable to maintain oral intake due to refractory nausea, debilitated medical status or extremes of age, or patients with refractory pain, hospital admission with urologic consultation may be required [24].

For all other patients, ambulatory management of renal calculi should be adequate. However, ambulatory management needs timely urologic consultation and close follow-up [24].

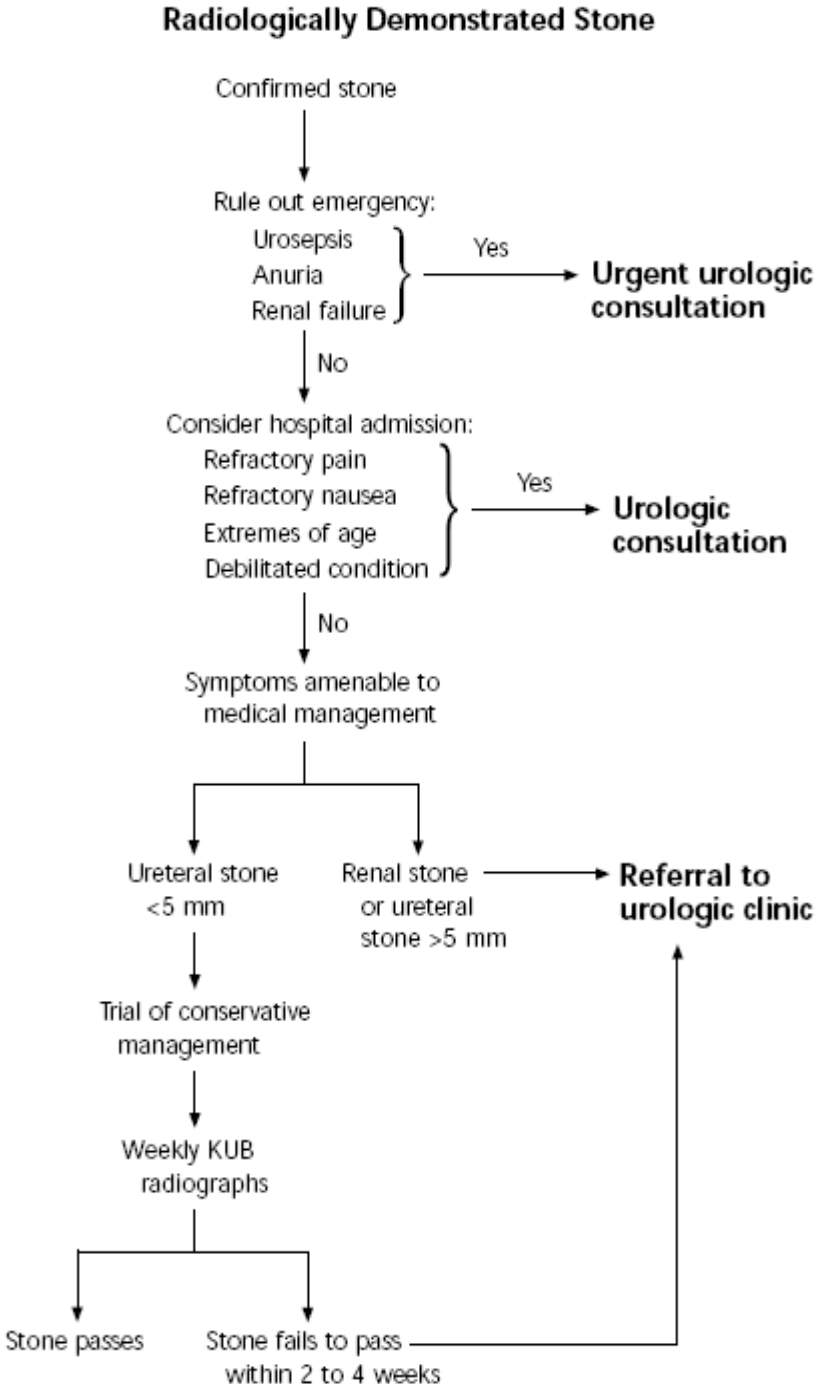


Figure 1.3. Management of urolithiasis. (Portis & Sundaram 2001)

1.3.4 Treatment for renal stones

Urinary stones that are less than 2 cm in size can generally be treated with extracorporeal shock wave lithotripsy (ESWL) [24]. However, stones in a lower pole calyx are associated with poor clearance rates after ESWL and thus 1 cm is the generally recommended upper limit for this treatment [25]. Larger stones are generally treated by percutaneous nephrolithotomy (PCNL).

Although ESWL provides a successful and non-invasive treatment for the patient to remove calculus, the recurrence rate following ESWL treatment remains high. About half of patients with previous urinary stone will have a recurrence within 10 years [26]. Most past stone-formers, if not all, after treatment are left with clinically insufficient residual fragments (CIRF) which are potential nucleators of new recurrent stone. However, there is no diagnostic marker to detect the presence of such nucleators or stones, although some urinary cytokines and urinary levels of mediators of inflammation are becoming increasingly important as markers of urological disease [27, 28]. Hence, studies of levels of mediators of inflammation may help in understanding the pathogenesis of urolithiasis and provide a reliable marker for early diagnosis, treatment and prevention.

1.4 Methods to study *in vitro* crystallization related to urolithiasis

Since renal stone formation starts from crystallization, study of *in vitro* crystallization can provide fundamental information to urolithiasis research. There are many different methods developed for qualitative and/or quantitative measurement of *in vitro* crystallization activity. Crystallization is a physicochemical process involving the transformation from liquid into a solid phase in a supersaturated solution. Three key factors (Figure 1.4) are involved in the development of crystallization [29]. First, the establishment of supersaturation is required for crystallization processes such as nucleation, growth and aggregation. Other physicochemical factors present can interact with crystallization. Second, supersaturation will decline (unless replenished) as crystallization proceeds and this results in the changes in the kinetic behaviour of the crystallization processes. Third, the processes of stone formation take place in a dynamic biological environment with a constant and continuous supersaturation.

Classification of crystallization methods [29] can be based on the type of process used, analysis parameters measured and the changes in the supersaturation profile during crystallization (Figure 1.5). Since the fundamental process for crystallization is supersaturation, the classification of crystallization methods in this review is based on the changes in supersaturation profile during crystallization.

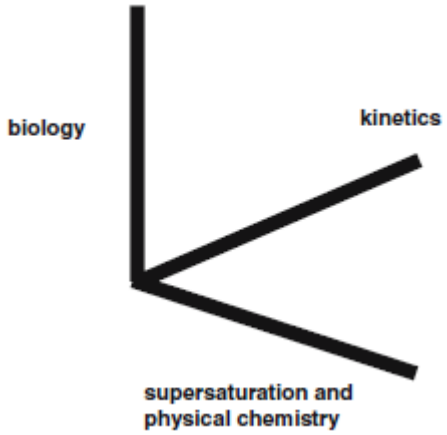


Figure 1.4. Three key factors involved in crystallization and stone formation. (Kavanagh 2006)

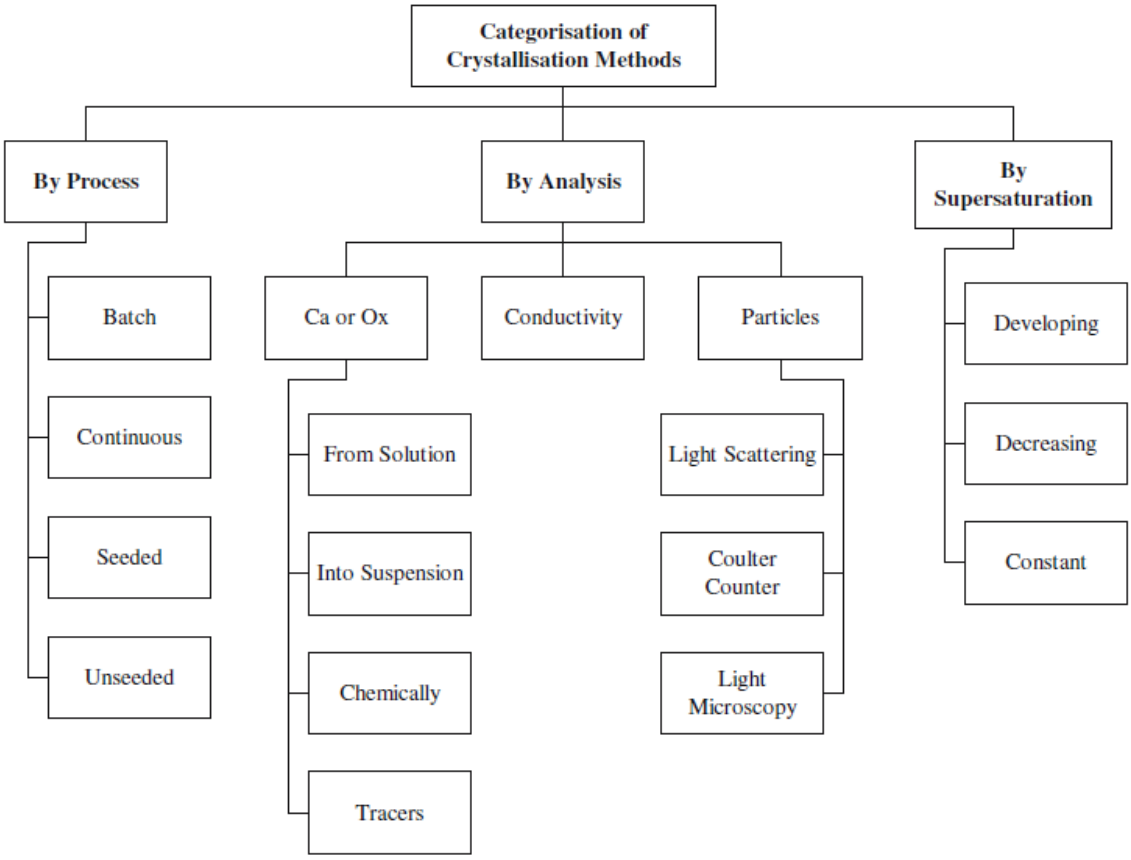


Figure 1.5. The classification of crystallization methods. (Kavanagh 2006)

1.4.1 Supersaturation decay systems

In supersaturation decay crystallization method, crystallization is induced and allowed to continue without any further additions and thus the supersaturation of the solution decreases as crystallization occurs.

1.4.1.1 Simple batch (discontinuous) systems

Simple batch (discontinuous) systems are widely used for crystallization studies because they are simple methods that just bring soluble salts of lithogenic ions such as calcium and oxalate solutions together to produce a supersaturated solution of CaOx when combined. Crystallization starts spontaneously when the supersaturation exceeds the formation product (FP) or metastable limit (ML), which is the upper limit of supersaturation dissolution. The supersaturation will reduce as calcium and oxalate are crystallized and removed from the solution until equilibrium with a supersaturation ratio of 1 is achieved. The occurrence of crystallization was usually observed by the change in turbidity or light scattering of solution. The kinetics of crystallization including the growth and nucleation of crystals were then estimated from the turbidity profile [30-33]. Crystals may be nucleating, growing and aggregating when the supersaturation decreases during crystallization. Thus, the amount of calcium or oxalate precipitated out from solution at a fixed time (or times) can be measured to estimate the growth of crystals [29].

The advantage of this system is that it is easy to perform, and the heterogeneous nucleating capacity of sample that leads to spontaneously precipitation of crystals as determined by ML [30-32, 34-36] makes this a quick and popular system for crystallization studies. Moreover, this method can be done by microplate and thus relatively small amount of modifier added into the concentrated calcium and oxalate solution is enough for the estimation of ML of crystallization.

However, this method involves the rapidly heterogeneous nucleation of suspension of crystals during crystallization that allows more crystals to grow so that the growth is not independent of the nucleation and aggregation, as aggregation will be affected by the crystal surface area presented to the solution. Therefore, it is difficult to separate the effects of different crystallization processes (nucleation, growth and aggregation) on crystals numbers and volumes even when the particle size distributions are measured and thus most experiments are performed as a simple comparison between the ML of crystallization of a test and control conditions rather than quantitating the nucleation, growth and aggregation of crystallization directly.

1.4.1.2 Seeded batch systems

In this system, seed crystals were introduced into a metastable supersaturated solution and allow the crystals to grow and aggregate. Quantitative results of crystallization including the solubility, growth, and agglomeration of crystals [37, 38] can be obtained by measurement of crystal size distributions since no nucleation

occurs in this system to interact with the aggregation and growth of crystals. However, this method is usually applied to the well-defined media such as artificial urine only since it is difficult to set up a well controlled metastable solution in whole urine due to the variability of compositions in each urine samples [34].

1.4.2 Supersaturation developing systems

This method is a relatively slow method for increasing the concentration of calcium and/or oxalate and sustaining it after crystallization was initiated. Although the supersaturation of solution can be continuously maintained when crystallization has started, the actual supersaturation of solution during the experiment is difficult to be defined.

1.4.2.1 Reverse osmosis and evaporation for supersaturation development

Reverse osmosis and evaporation methods [39-42] were specifically developed to mimic the water re-absorption in the upper urinary tract so that urine will become supersaturated for crystallization. Evaporation methods are simple methods where urine is evaporated at 37℃ at a fixed osmotic pressure to induce crystallization [39] and the crystals formed were examined microscopically. Whereas reverse osmosis is an advanced method that calcium and oxalate solutions below supersaturation were fed into hollow fibre reverse osmosis membranes that are permeable only to water [43] at high pressure so that supersaturation can be developed to induce crystallization.

Since this method is a continuous flow system, it can mimic the transit times through the renal tubules by adjustment of the transit times through the membranes to around 3 minutes so that nearly 80% of water was reabsorbed to generate supersaturation to induce crystallization. The crystals numbers, sizes as well as extent of aggregation are examined by scanning electron microscopy [43].

These supersaturation developing methods can be used for the comparison of the crystallization properties of fresh urine between stone formers and normal individuals [39, 42]. However, each urine will vary depending on their initial saturation and osmotic pressure that leads to variable results. Urinary macromolecules (UMMs) recovered after ultrafiltration of urine and other modifiers including urinary inhibitors can be added to this system to study their effects on crystallization [40, 43-48]. Besides, different formulas of artificial urine or modified human urine can be used to mimic the concentrations of urine in different regions of the renal tubules [49-51] for crystallization studies. However, it cannot analyze nucleation, growth and aggregation crystallization processes at the same time independently.

1.4.2.2 Gel diffusion of calcium and/or oxalate for saturation development

Calcium and oxalate are allowed to diffuse slowly towards each other through an agar gel that leads to crystallization at the boundary where there they meet. The development of supersaturation at the boundary leads to relatively slow growth of crystals that can allow enough time for them to give rise to large crystals for

structural or morphological examination. Crystallization can be quantified by enhancement in turbidity [52] and urine inhibitors and other modifiers can be added and allowed them to diffuse into the crystallization zone [53] to study their effects on crystallization. This method can therefore become easily adapted to study many different samples by just adding them into gel and let them diffuse through the gel. However, the relative diffusion rates of inhibitors such as different types of UMMs may be different and may lead to the comparisons of these samples to become difficult. Besides, this method can only measures the changes in turbidity for studying of crystallization and thus the detailed study on the crystallization parameters such as nucleation, growth and aggregation of crystals become impossible.

1.4.3 Constant supersaturation systems

The urinary system is a continuous flow system for which the saturation in each region maintains constant since the contents within each region are being continually replenished. Thus, crystallization in a supersaturated urine will not affect the supersaturation of urine by continuous replacement of the lithogenic ions. Researchers in urolithiasis studies have therefore developed two widely used systems in order to mimic the urinary system. They are the constant composition system and the mixed suspension, mixed product removal (MSMPR) system.

1.4.3.1 Constant composition system

This system uses a metastable solution seeded with crystals [54-57] to study crystal growth or unseeded mode to examine nucleation [51, 58, 59] of crystals. The growth of crystals in the seeded method leads to the reduction of the lithogenic ions such as calcium and oxalate in equimolar proportions. A calcium electrode was used to detect the reduction in ionized calcium and this leads to a feedback control for which a syringe pump will response to the decline of lithogenic ions by adding calcium and oxalate solutions (Figure 1.6) in order to restore the balance of ions to the original level. Therefore, the lithogenic ions can maintain at a constant level throughout the experiment. The growth rate [57, 60] of the seeded crystal can be estimated by the rate of pumping of new reactants. Whereas in unseeded method, nucleation occurs in naturally unstable metastable solutions that leads to crystallization. Thus, unseeded method can be applied to study the effects of medium such as human urine on the nucleation of crystals by measuring the lag time (the induction time) required (Figure 1.6) for crystallization to occur.

This system can maintain a constant supersaturation for crystallization to occur and either growth rate in seeded method and nucleation rate in unseeded method can be studied. However, crystals concentration is continuously increased in this system and this may lead to crystal aggregation that may indirectly affect the growth rates of crystals.

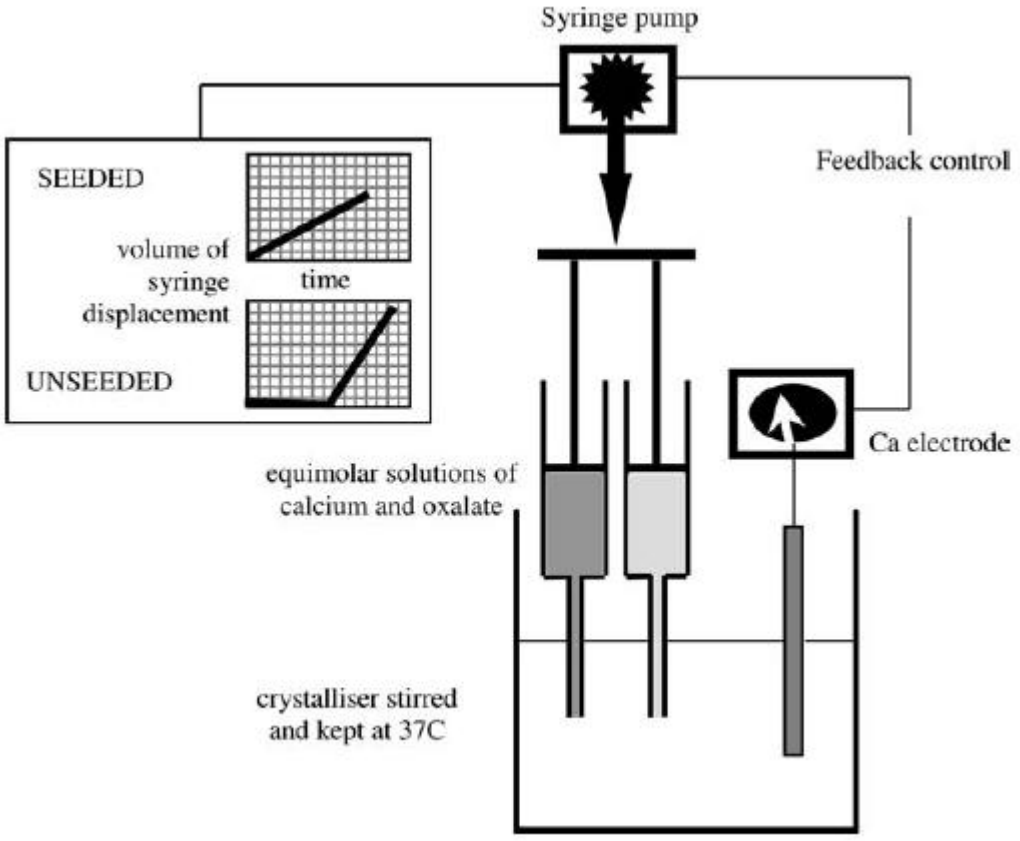


Figure 1.6. Constant composition system. The growth of seeded crystal in seeded method and the nucleation of crystals in unseeded method lead to the reduction in the ionized calcium that is detected by the calcium electrode and responded by a syringe pump to replace calcium and oxalate solutions to the original level. (Kavanagh 2006)

1.4.3.2 Mixed suspension, mixed product removal (MSMPR) system

The urinary system is a continuous flow system with fresh supersaturated urine being continuously formed and passed out of the kidney and urinary tract [10]. Therefore, this system aims to mimic the continuous flow urinary system for which a continuous input of reactants (lithogenic ions) in a supersaturated solution such as artificial urine (AU) and removal of mixed product suspension so that the saturation in the system maintains constant by continual replenishment of lithogenic ions. A MSMPR system (Figure 1.7) consists of a well mixed chamber with input tubings of lithogenic ions such as calcium and oxalate solutions as well as a supersaturated solution such as AU to induce crystallization, and also an output tubing for mixed product removal that is adjusted for maintaining the constant volume in the chamber [10]. A characteristic residence time (volume/flow rate), which is the average time of crystals remaining in the crystallizer, was defined on each system based on the chamber volume and flow rate of the system. A dynamic equilibrium with a stable supersaturation and crystal size distribution of the MSMPR system can be developed after 6-8 residence times. The crystallization kinetics including the nucleation and growth rates [34] of crystals are then calculated from the crystal size distributions that can be measured by a particle size analyzer.

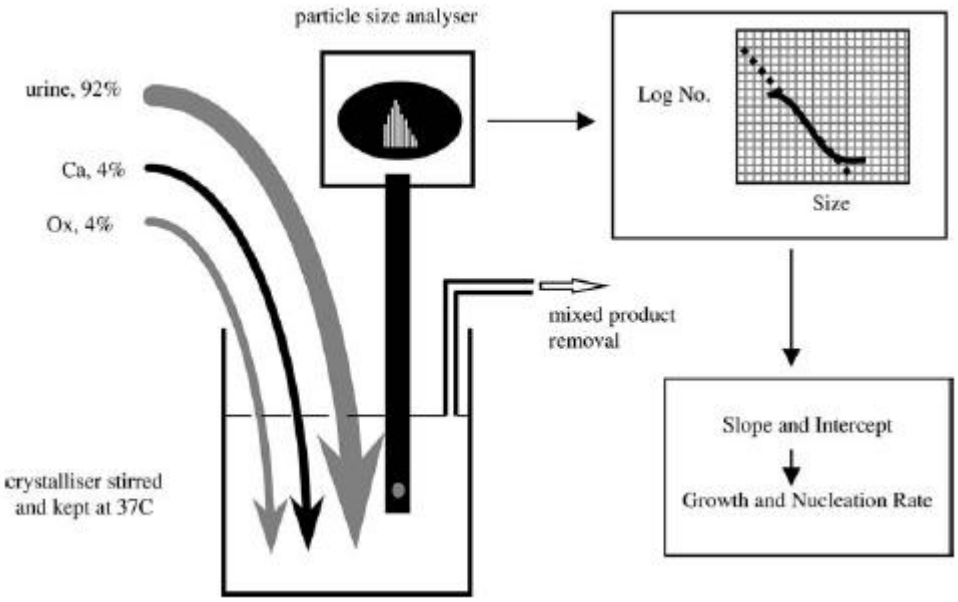


Figure 1.7. The mixed suspension, mixed product removal (MSMPR) system. Lithogenic ions and supersaturated solutions were added into the crystallizer of the system to induce crystallization and same volume as the input solutions was being removed as mixed product removal for maintaining constant volume and supersaturation within the crystallizer of the system. The crystals formed, after 6 to 8 residence times to reach the equilibrium of the system, were analyzed by a particle size counter and plot with Ln (N) against crystal sizes. Growth and nucleation rates of crystallization were calculated from the slope and y-intercept of the plot, respectively. (Kavanagh 2006)

Since aggregation of crystals may happen during the crystallization process that can interact with the nucleation and growth of crystals in the MSMPR system, a plot of Ln (N) against crystal sizes is performed (Figure 1.8) for excluding the presence of crystal aggregation. A linear plot of Ln (N) against crystal sizes with a high r^2 (>0.95) analyzed by linear regression represents a realistic growth and nucleation rates of crystals without being affected by the aggregation of crystals.

The slope and y-intercept of the plot (Figure 1.9) were used for the calculation of the growth and nucleation rates of crystallization, respectively by the following equations from previous studies [5, 61].

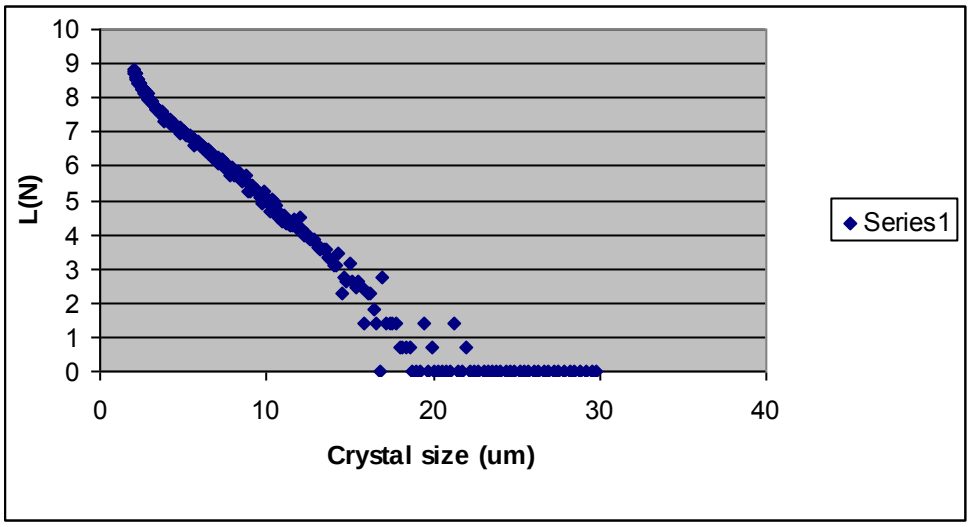


Figure 1.8. A plot of Ln (N) against crystal sizes (μm) of crystals with high r^2 analyzed by linear regression represents a realistic growth and nucleation rates of crystals without being affected by the aggregation of crystals.

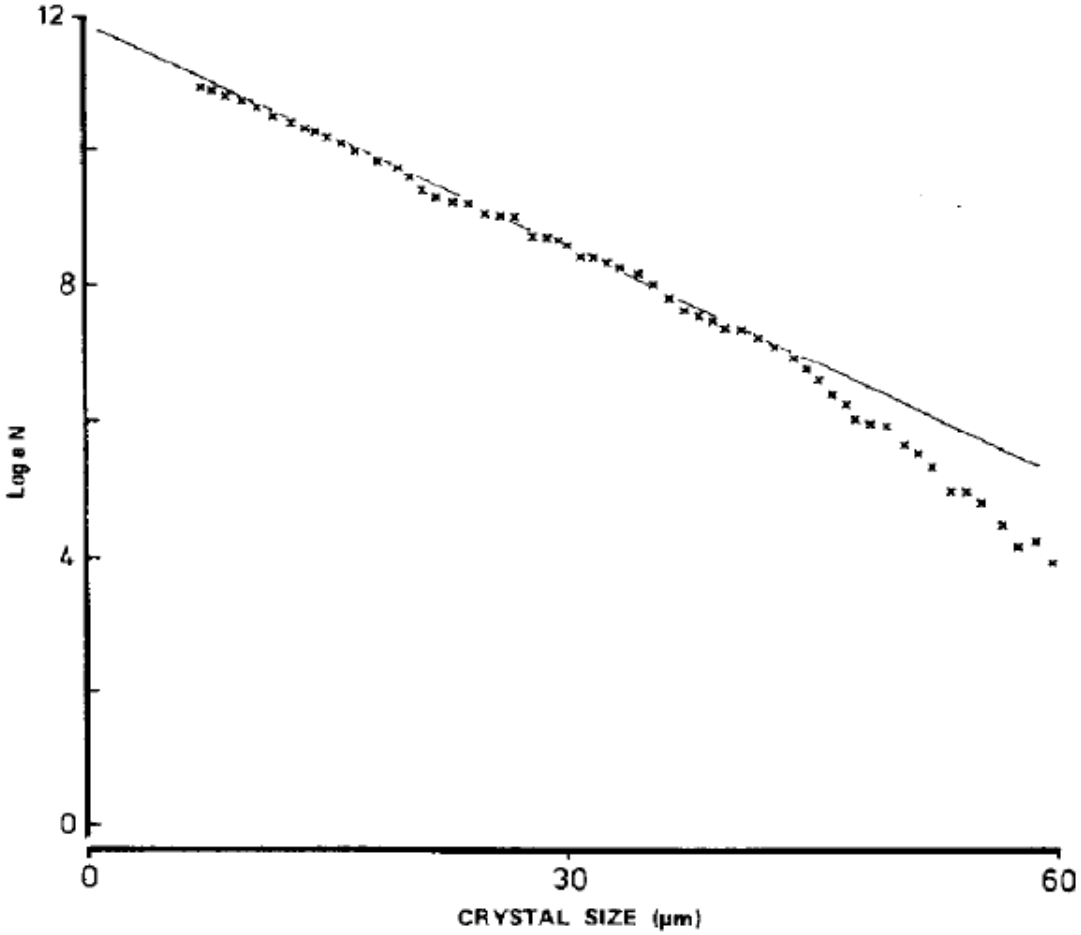


Figure 1.9. A linear plot of Ln (N) against crystal sizes (μm) of crystals gives the slope and y-intercept for the determination of the nucleation and growth rates of crystallization. (Kavanagh *et al* 1991)

The mean residence time (τ) is the average time that crystals remain in the crystallizer and is given by $\tau = V/Q$ where,

V = chamber volume

Q = total flow rate

$N = (B_o/G) \exp(-L/G\tau)$ or $Ln(N) = Ln(B_o\tau) - L/G\tau$ where,

N = population density (numbers/mL)

L = crystal size (μm)

B_o = nucleation rate (numbers/minute/mL)

G = growth rate (μm/minute)

τ = mean residence time (minutes)

$Ln(N) = Ln(B_o\tau) - L/G\tau$

Intercept (I) = $Ln(B_o\tau)$

B_o = Anti Ln (I/τ)

Slope = $-1/G\tau$

G = -1/Slope τ

The suspension density (M_T, mmol/L or mM) can be calculated from the G and Bo according to the following equation:

$M_T = \pi\rho BoG^3\tau^4 / D \times 10^{-6}$ where,

$\pi = 3.14$

ρ = crystal density (g/cm^3)

D = molecular weight of crystal

Thus, MSMPR system is an unique system that can allow the measurement of growth and nucleation rates as well as suspension densities of crystals at the same time independently and thus this system is chosen for the investigation of kinetics on crystallization in this study.

1.5 Modulators of crystal and stone formation

Urinary modulators can be classified as low molecular weight (M.W.) compounds such as citrate and high M.W. compounds such as glycoproteins (GPs), glycosaminoglycans (GAGs) and others urinary macromolecules (UMMs). They modulate crystallization and/or crystal retention in the urinary tract that leads to renal stone formation either by directly interaction with crystals or indirectly influencing the urinary environment.

1.5.1 Low M.W. compounds related to stone formation

1.5.1.1 Citrate

Citrate is the most abundant anion present in urine and it can bind calcium to from a complex and thus reduce the nucleation [62] and growth [63] of calcium crystals [64]. Study from Kok *et al* [65] showed that recurrent stone formers with low citrate excretion lead to excessive crystal agglomeration. Therefore, the reduced excretion of urinary citrate (hypocitraturia) is a significant risk factor for calcium containing stone formation [66]. Many studies reported that calcium stone formers suffered from hypocitraturia [67-77]. Therefore, citrate supplementation is one of the therapeutic agents for the treatment of calcium renal stones. Potassium citrate is not only used for the correction of hypocitraturia that aims to treat calcium stones, but also corrects low urinary pH [78] due to the alkalization of urine by citrate. A meta-analysis study from

Hess *et al* showed that potassium citrate administrated orally enhanced the urinary pH and citrate by 14 and 94%, respectively [79] and significantly reduced the stone formation rate by 47-100% [79]. Citrate can also decrease the crystal-cell interactions by reducing half of the binding of CaOx crystals to renal epithelial BSC-1 cell line [80].

1.5.1.2 Magnesium

Magnesium is an inhibitor of CaOx crystallization due to its chelation effect by forming ion complexes with oxalate and being more soluble. Studies found that magnesium can inhibit the nucleation [81] and growth [62, 81] of CaOx crystals and reduce CaOx crystallization in human whole urine [82]. Magnesium can inhibit the growth and aggregation of both CaOx and calcium phosphate (CaP) crystals *in vitro* [83]. However, studies found that there was no difference on the urinary magnesium excretion between stone-formers and normals [84, 85] and magnesium oxide therapy did not show any therapeutic effect in recurrent calcium stone formers [86].

1.5.2 High M.W. compounds related to stone formation

1.5.2.1 Nephrolithiasis-related UMMs

Nephrolithiasis-related UMMs are usually present in renal stones, are expressed and synthesized in the kidney, and are involved in modulation of the crystal nucleation, growth or aggregation that eventually affect the renal stone formation [87]. Many

nephrolithiasis-related UMMs (Table 1.1) are produced in the kidney as a response to tissue damage [87] including urinary proteins (GPs) such as prothrombin (PT), osteopontin (OPN), Tamm-Horsfall protein (THP), inter-α-trypsin inhibitor (ITI), bikunin and urinary glycosaminoglycans (GAGs) such as chondroitin sulfate (CS) and hyaluronan (HA) [87].

Table 1.1. Nephrolithiasis-related urinary macromolecules. (Verkoelen & Schepers 2000)

Name	Abbrev-iation	Tissue repair	Crystallization
Prothrombin family of proteins	PT PT-F1 PT-F2 Thrombin	Thrombin plays an important role in tissue repair	PT-F1 is an inhibitor of crystallization
Inter-α-trypsin inhibitor Family of proteins	ITI ITI-HC1 ITI-HC2 Bikunin	ITI-HC stabilize the pericellular matrix surrounding wounded areas	Bikunin is an inhibitor of crystallization
Osteopontin	OPN	OPN is a chemoattractant for monocytes during inflammation	OPN is a modulator of crystallization
Tamm-Horsfall protein	THP	THP interacts with neutrophils during inflammation	THP is a modulator of crystal aggregation
Chrondroitin sulphate	CS	CS is the major urinary GAG	CS is not present in the organic kidney stone matrix
Hyaluronan	HA	HA plays an important role during renal injury and repair	HA is the major GAG in the organic kidney stone matrix

CS, chondroitin sulphate; HA, hyaluronan; HC, heavy chain; ITI, inter-α-trypsin inhibitor; OPN, osteopontin; PT, prothrombin; THP, Tamm-Horsfall protein.

1.5.2.2 Urinary GPs in renal stone formation

There are plenty of studies about the effects of different urinary GPs on the CaOx crystallization. THP is the most abundant protein in normal human urine [88]. However, it is absent in CaOx crystals and only trace amounts of THP was found in the stone matrix [89]. A study demonstrated a promotory effect of THP on CaOx crystallization by enhancing the precipitation of CaOx [44] in concentrated urine, but another study showed inhibitory effect of THP on CaOx crystal aggregation while no effect on the nucleation and growth of crystals [90].

Other urinary GPs have also been reported with specific roles on the CaOx crystallization. Bikunin produced by kidney [91] and enhanced expression of bikunin mRNA was observed in renal epithelial cells exposed to oxalate and CaOx crystals [92, 93] and it is a potent inhibitor of CaOx crystallization by inhibition of nucleation and growth of CaOx crystals [94].

Increased expression of OPN was observed when renal tissue was damaged by CaOx stones or crystals [95] and OPN is a potent inhibitor of nucleation [96], growth and aggregation [97] of CaOx crystals. Urinary albumin is present in urine [98, 99] and also detectable in the matrix of urinary stones [99] and crystals [100]. Studies have shown that albumin can bind to CaOx crystals [101, 102] but no inhibitory effect on CaOx growth [90, 101]. Other studies have shown the promotory effect of albumin on the crystal nucleation [103].

The contradictory results within and between each individual urinary GPs make the explanations of the actions of urinary GPs on CaOx crystallization becoming complex, and thus requires further elucidation. Besides, the studies are specific to one or two GPs and ignores any contribution of other GPs as a whole. Thus, we performed an experiment to study the effect of total urinary GPs on the CaOx crystallization by an in-vitro MSMPR system.

1.5.2.3 Urinary GAGs in renal stone formation

GAGs are one of the major classes of macromolecules that play an important role on the CaOx crystallization. They are a group of polysaccharides with repeating disaccharides units, in which one of the sugars is either N-acetylgalactosamine or N-acetylglucosamine or one of their derivatives [104]. GAGs are highly negatively charged because of the presence of carboxyl or sulphate groups on their sugar residues that lead to the stretched or extended conformation of polysaccharide chains of GAGs.

Urinary GAGs can be derived from filtration by the glomerular basement membrane, shedding from the surface of the tubular epithelial lining and the urothelium of the urinary tract [105]. Some studies showed that the urinary excretion of GAGs was significantly lower in stone formers than in normal individuals [106-110] while other studies demonstrated no significant differences between them [111, 112]. There are five types of GAGs that are known to exist in urine including heparan sulphates (HS), dermatan sulphates (DS), chondroitin sulphates (CS), hyaluronic acid

(HA) and in trace amounts keratan sulphate (KS). GAGs have also been isolated from the matrix of urinary stones [113] and results showed that HA was found in apatite and struvite stones, HS in calcium oxalate monohydrate and uric acid stones and HA and HS in calcium oxalate dihydrate stones.

Table 1.2. Urinary excretion of various GAGs. (Khan & Kok 2004)

Compound	**Average urinary excretion, mg/24h (range)**	
	Control	**Stone Former**
Total GAGs	23-28 (0-50)	23 (0-53)
CS	14 (8-18)	14 (4-19)
HS	5 (2-14)	5 (3-14)
HA	3 (1.8-7.5)	3 (1-8)

GAGs, glycosaminoglycans; CS, chondroitin sulphate; HS, heparan sulphate; HA, hyaluronan.

The increase production of GAGs by tubular epithelial cells may protect cells from toxic effects of calcium oxalate crystals and oxalate ions [114]. However, the role of GAGs on CaOx crystallization is still controversial since both inhibitory and promotory effects on crystallization were reported in several studies [40, 109, 115-118].

1.5.2.4 The role of HS in renal stone formation

HS is the major GAG that can be extracted from stone matrix material [113, 119]. HS enhanced the nucleation rate [106] of CaOx crystals and the material extracted from stones, presumably containing HS, had been shown to enhance the nucleation rate [106] of CaOx crystals and inhibit crystal growth [106] in urine. It may be due to its chemical structure [120] such as its peptide content, length of the carbohydrate-peptide linkage region, degree of sulphation, or its molecular size that enhances its adsorption onto crystals.

1.5.2.5 The role of CS in renal stone formation

CS is the major GAG present in human urine [61, 121]. CS inhibits crystal nucleation rate but promotes growth rate of crystals [61, 122]. Since CS is absent in both the CaOx crystals and stone matrix [113, 123], it is hypothesized that the action of CS in CaOx crystallization may be related to the interaction between CS to others modulators for CaOx crystallization rather than directly acting on the crystals or stones.

1.5.2.6 The role of HA in renal stone formation

HA, being part of the glycosaminoglycan family, is a nonsulfated, negatively charged linear polysaccharide (Figure 1.10) composed of repeating disaccharide units of (β1→4)-D-glucuronic acid-(β1→3)-N-acetyl-D-glucosamine [124].

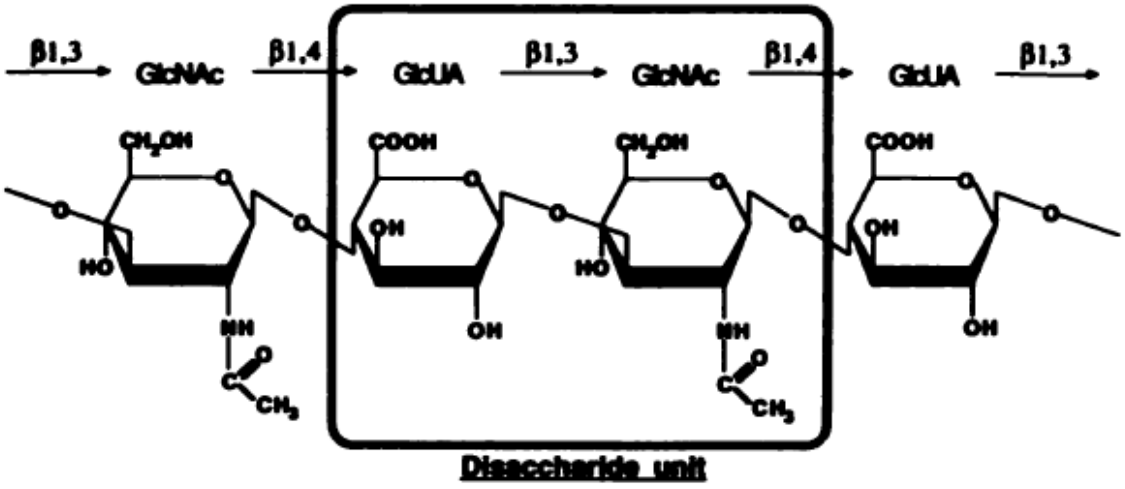


Figure 1.10. The chemical structure of hyaluronan. (Laurent & Fraser 1992)

HA has been found in all mammalian tissues including the connective tissue, extracellular matrix and synovial fluid [124]. HA is involved in several fundamental cell biological processes such as regulation of cell-cell adhesion, development, proliferation, migration, differentiation, metastasis, inflammation, and wound healing [124]. It is an ubiquitously distributed component of the extracellular matrix and in its native form exists as a high molecular weight (M.W.) polymer, in excess of 10^6 Daltons [125].

HA is the major polysaccharide component of the organic kidney stone matrix [126]. It is found to be the major contributor to the crystallization-promoting property of the renal stone formation. Urinary HA is excreted in a significantly higher proportion (11.6% of total GAGs) from stone-formers than HA in the urine of normal subjects (6.2%) [115]. It maybe a consequence of active turnover of renal tissue in the diseased state during which hyaluronan fragments are released into urinary tract [127]. Studies showed that migrating cells produce large amounts of HA during repair of damaged renal epithelial cells [128, 129].

HA might participate in one or more stages of renal stone formation (Figure 1.11). The multiple roles attributable to HA in renal stone formation might be a consequence of the various size- related function of HA [115]. It is usually found in the medullary and papillary interstitum of the normal kidney as a high M.W. species [130], but urinary HA is predominantly of a lower M.W. of about 10kDa [131]. High molecular mass species of HA can coat crystals and block the crystal-crystal or crystal-cell

interaction, whereas low molecular mass species become associated with the cell surface, mediate crystal adherence and crystal nucleation by binding to the cell surface HA-binding proteins such as CD44 [115]. Another study suggested that hyaluronan once attached onto cell can prevent cells, particles and large molecules from approaching closely to the cell membrane and high molecular mass hyaluronan is a more efficient shield than low molecular mass hyaluronan at similar concentrations [132]. An enzyme, glucose oxidase, which is generated from damaging free-radicals including OH^- radical in a Fenton reaction was tested in a study. High molecular mass hyaluronan was an effective shield against the product of the enzyme, significantly diminishing cell damage, compared with low molecular mass hyaluronan [132].

The production of high molecular mass of HA during epithelial injury will be degraded to lower molecular mass HA fragments under the influence of inflammation, oxygen free radicals or specific hyaluronidases [133, 134]. These low molecular mass HA are released into the tubular lumen of urinary tract and participate in promoting the CaOx crystals growth and aggregation, as a coincidence rather than a cause, leading to renal stone formation. Moreover, HA has been found to bind calcium oxalate crystals at the surface of proliferating renal tubular cells in cell culture model. It also acts as a crystal-binding molecule at the surface of Madin-Darby canine kidney (MDCK) cells by the adherence of calcium oxalate mono-hydrate crystals to the renal tubule epithelium – an event considered to be a critical event in the pathophysiology of calcium nephrolithiasis [87].

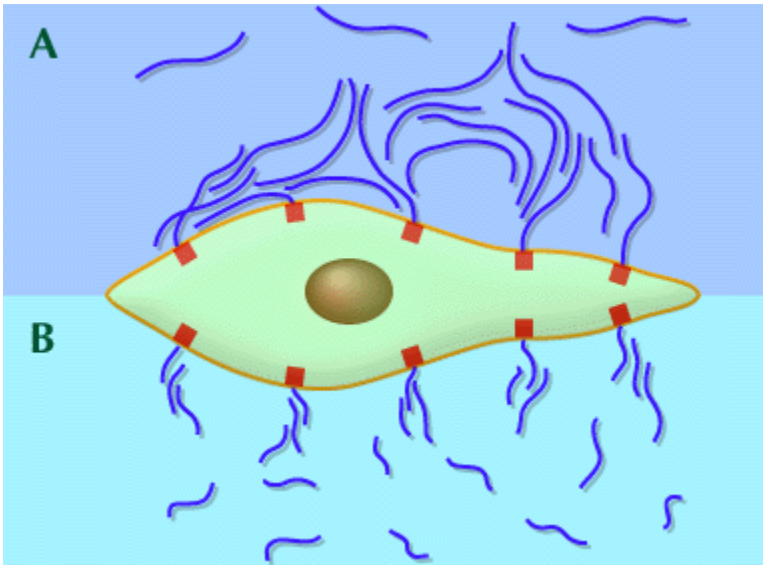


Figure 1.11. (A) High molecular mass hyaluronan meshworks overlap and aggregate with hyaluronan molecules attached to adjacent cell surface binding sites.
(B) Low molecular mass meshworks contain hyaluronan molecules which are not long enough to overlap and reinforce adjacent hyaluronan aggregates, leaving spaces and channels through which particles can approach closely to the cell membrane. (Scott 1998)

1.5.2.7 CD44 – a HA-binding protein on surface of cells

CD44 is a major HA receptor which is expressed by a wide range of cell types, including lymphocytes, macrophages, tubular epithelial cells, fibroblasts and mesangial cells [135-140]. Low molecular weight HA-CD44 interactions are involved in serveral immune responses discussed in the next section.

1.5.2.8 The interactions between HA and CD44

Low molecular weight HA-CD44 interactions (Figure 1.12) can activate peripheral blood T-cells, resulting in the stimulation the secretion of IL-2, and promote the expression of the pro-inflammatory cytokines, IL-1 and TNF-alpha by monocytes [137]. The influences of HA-CD44 interactions are not limited to the release of cytokines, as chemokines Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein (MIP-1α and MIP-1β) are also activated due to the binding of HA to macrophages [137, 141].

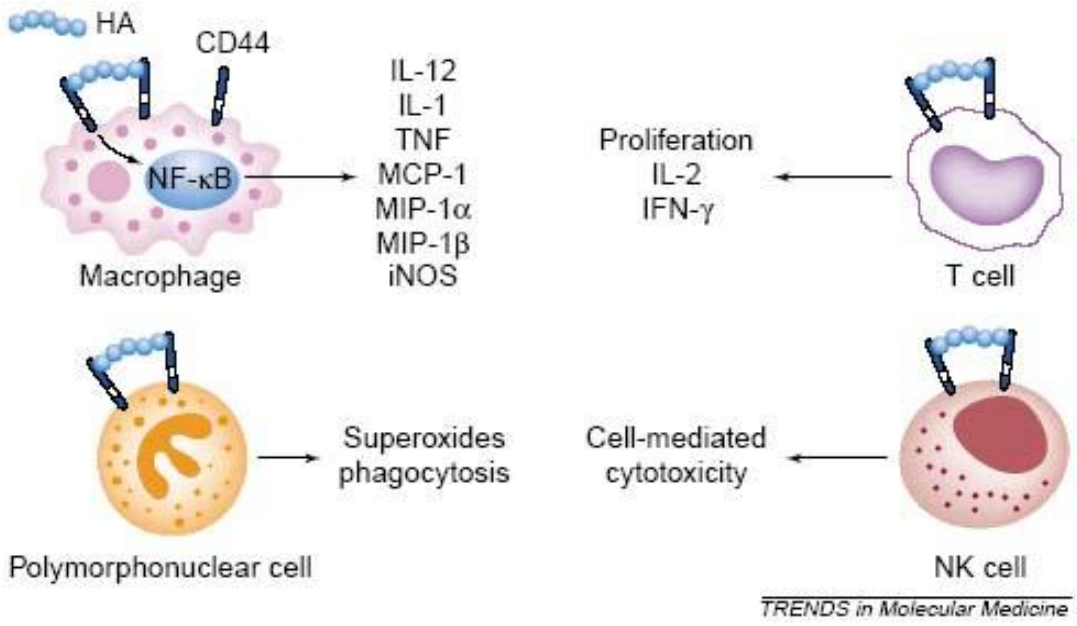


Figure 1.12. HA-CD44 interaction mediates different leukocytes activation. (Pure & Cuff 2001)

A study on the role of HA fragments size and CD44 on chemokine gene expression was demonstrated in the murine alveolar macrophage cell line (MH-S) and human monocytic leukaemia cell line. The results demonstrated that HA fragments were generated from high molecular weight HA during inflammation, which induced the expression of macrophage genes by HA binding in part through CD44 in MH-S cells [142]. Moreover, aggregation and extravasation of leukocytes including lymphocytes and macrophages to the sites of inflammation are induced by HA-CD44 interaction [140].

Another study showed the HA-CD44 interaction mediates the adhesion of activated T-lymphocytes to human gingival fibroblasts. The phorbol 12-myristate 13-acetate (PMA) activated T cell which were stained by immunofluorescence showed a strong adhesion to the human gingival fibroblasts culture plate which was coated with HA but inhibited by addition of OS/37 which was a monoclonal antibody specific for the hyaluronate-binding epitope on CD44 [143].

This CD-44-HA-CD44 interaction is thought to occur by one cell presenting surface-bound HA anchored by its cell surface CD44 and then being recognized by CD44 on the opposite cell. Thus, HA can act as a bridge to mediate CD44-HA-CD44-dependent cell adhesion [144].

1.6 Novel renal stones discovered in China – melamine stones

During the period that the urinary factors which affect the physiochemical properties of CaOx crystallization and its effects to renal stone formation were being studied, increased incidences of renal stone disease occurred in Chinese infants in early September 2008 lead to our concern on how renal stone disease were being formed in infants. Subsequently, melamine was found added into infant milk powders in order to substitute for the protein contents and fed to infants. Infants with intake of melamine-contaminated milk developed urinary tract diseases such as acute renal injury due to the blockage of urinary tract by melamine stone. Unlike CaOx crystallization, the physicochemical nature of melamine in urine and its handling were largely unknown at that time. Therefore, we studied the melamine crystallization based on the previously established MSMPR system.

1.6.1 Renal stones in Chinese infants due to melamine

Since 2004, acute renal failure in animals was found to be associated with adulterated pet food in Asia and United States [145]. Melamine was found contaminated in the wheat gluten added to pet food. A co-contaminant, cyanuric acid was also identified [146]. In early September 2008, there were increased incidences of renal stones in infants and children, admitted in hospitals in China. Subsequently, melamine was found added into milk, in an attempt to increase nitrogen content (66.6% by weight) during classic crude protein test (Kjeldahl and Dumas method) for

dairy products so as to increase the measured protein content. At the time, 6 infants were reportedly to have died and around 300,000 suffered from urinary tract ailments including kidney stones, 850 are still being treated and 150 are seriously ill [147]. In Hong Kong, 15 children had tested positive and over 40,000 had been screened to be healthy [148, 149]. A study of 3,835 children attending Princess Margaret Hospital in Hong Kong were further investigated and 22 (0.6%) showed renal disorders but not necessarily related to melamine [150]. Most children afflicted with melamine-related stones were described as asymptomatic [151] until renal abnormalities were severe enough to cause impaired renal function by which time melamine and its crystalline stone had done its damage. Hence, the physicochemical nature of melamine in body fluids (blood and urine) and its handling is largely unknown until recently. Recent reports suggest that the levels of melamine and cyanurate allowed by the World Health Organization (WHO) should be lowered for infants (< 3-years old), as the relative risk for renal stones was 1.7 compared to control at the previously defined safe levels (< 0.2 mg/kg per day) [152].

1.6.2 Melamine crystallization

Melamine is a nitrogen-rich heterocyclic synthetic chemical (Figure 1.13) with a variety of industrial uses including the production of resins and foams, cleaning products, fertilizers and pesticides [153]. It does not occur naturally in food. In toxicological studies, melamine alone does not cause renal damage [154]. Melamine is soluble in water (3240 mg/L) [155]. However, when melamine is combined with cyanuric acid, insoluble crystals will form and obstruct renal tubules [145, 146]. Whilst most infants and children affected are less than 3-years old, the long-term effects and damage are not known. This study purports to investigate melamine and cyanurate toxicity in terms of crystallization that leads to future stone.

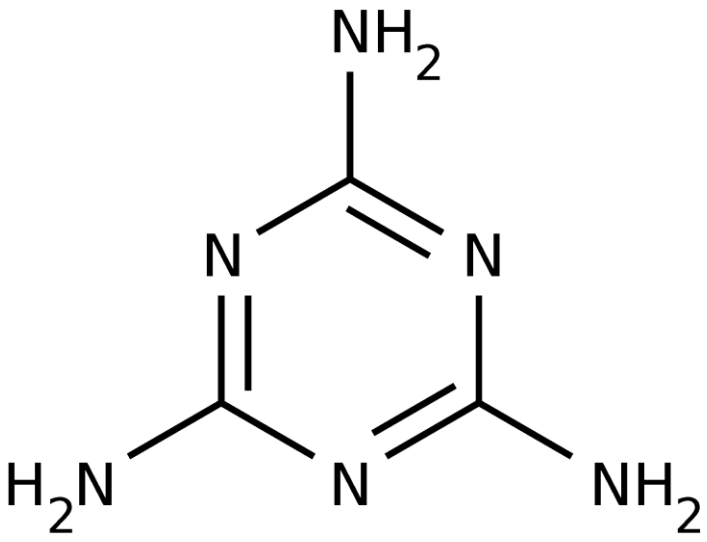


Figure 1.13. The nitrogen-rich heterocyclic structure of melamine. (Luigi Chiesa 2008; http://en.wikipedia.org/wiki/Melamine)

1.6.3 Cyanuric acid – a melamine analogue

Cyanuric acid is a cyclic chemical exists with enol and keto forms (Figure 1.14) that are intercovertable by tautomerization. It is prominent in enol form in water due to the stabilization of the presence of aromatic ring. Cyanuric acid is soluble in water (2000 mg/L) [155]. It is a by-product from melamine production and can react rapidly with melamine to from melamine cyanurate complex which locks cyanuric acid in the keto forms and become insoluble in water.

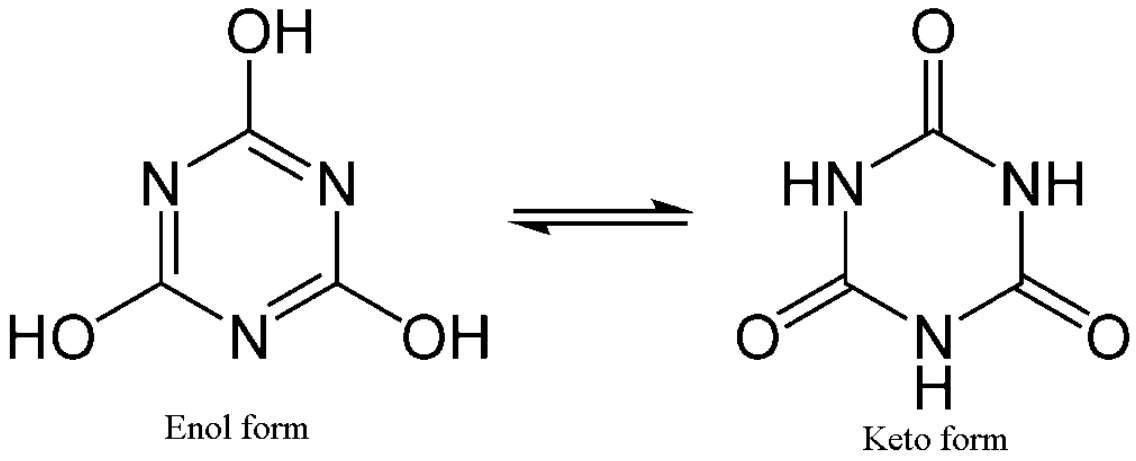


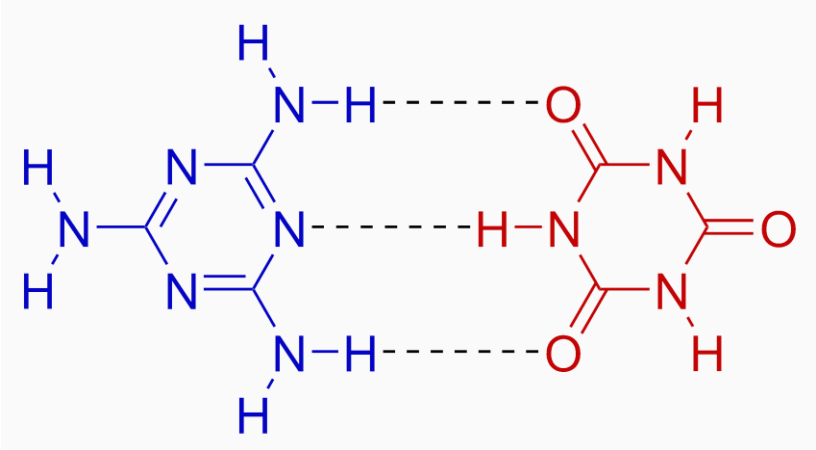
Figure 1.14. Cyanuric acid isomers.

(Yikrazuul 2008; http://en.wikipedia.org/wiki/Cyanuric_acid)

1.6.4 Melamine cyanurate crystallization

Once melamine and cyanuric acid combine, they form insoluble melamine cyanurate complex (solubility in water being 2 mg/L) [155] that is held by hydrogen bonding (Figure 1.15A). Therefore, the ratio of melamine to cyanuric acid is 1:1 for melamine cyanurate formation [156]. This hydrogen bonding can be multiplied between each melamine cyanurate complex that leads to the lattice assembly structure [156] consisting of alternating melamine and cyanuric acid units (Figure 1.15B).

A



B

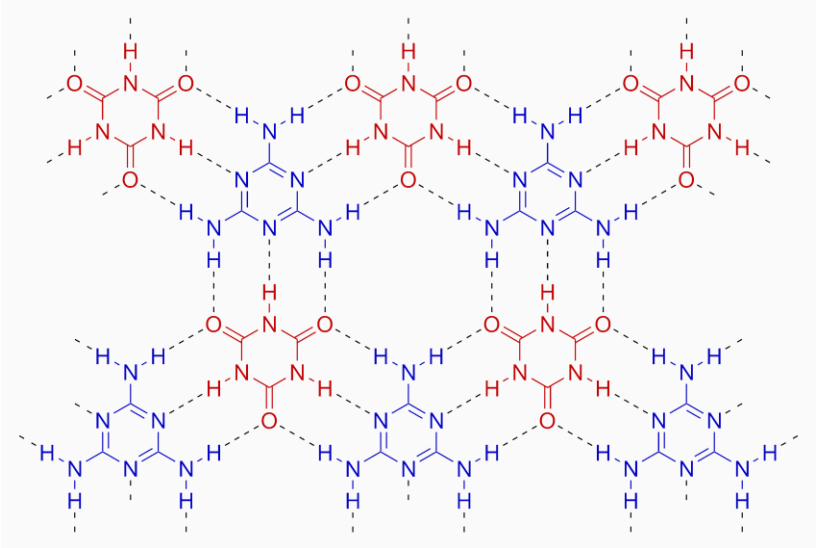


Figure 1.15. Melamine cyanurate crystallization. (A) Melamine and cyanuric acid combine together to melamine cyanurate complex by hydrogen bonding and (B) the multiples of hydrogen bonding between complexes leads to lattice assemble of insoluble melamine cyanurate crystals.

(Cacycle 2008; http://en.wikipedia.org/wiki/Melamine_cyanurate)

1.6.5 Melamine nephrotoxicity

Melamine is excreted rapidly (90% being excreted after 24 hours of administration) in rat urine [157] and thus melamine or its analogue – cyanuric acid alone is not enough to cause nephrolithiasis as evidenced from animal studies of fish, pigs [158], cats [146], and rats [159]. These studies indicated that both melamine and cyanuric acid should be present at the same time for the melamine cyanurate crystallization to occur both *in vivo* and *in vitro*.

There are some susceptibility factors that favour the melamine crystallization (Figure 1.16). These including the presence of cyanuric acid, the reduction in the urine volume, the low urine pH and uricosuria [160]. The presence of cyanuric acid leads to the formation of melamine cyanurate complexes in lattice assembly structure that are insoluble in urine. The reduction in the urine volume enhances the urinary supersaturation and melamine become more easily to precipitate in urine as melamine crystals.

Like other renal stone diseases, melamine stone formation needs the crystal deposition, attachment on the urinary epithelial cells and growth to a full-size stone to become pathogenic. The melamine stone formation eventually leads to inflammation of urinary epithelial lining, distal tubular necrosis and corticomedullary hemorrhage [160]. Thus, melamine nephrolithiasis at the same time causes the acute renal injury and the renal abnormalities are severe enough to cause impaired renal function and

become symptomatic for the stone patients. Recurrence of the melamine stones can result in chronic renal disease.

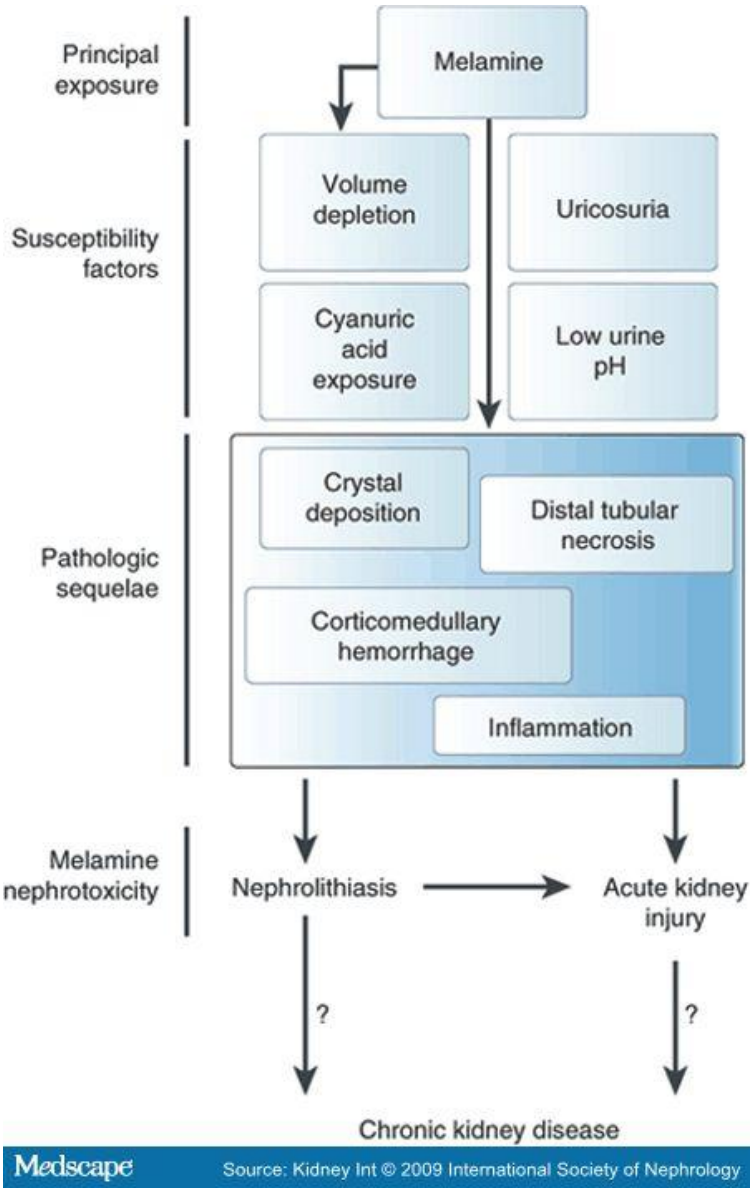


Figure 1.16. Possible mechanisms of melamine nephrotoxicity. (Bhalla *et al*, 2009)

1.6.6 Physiochemical properties of melamine crystallization

There are some fundamental questions such as what is the optimal proportion of melamine and cyanurate, at what pH and ionic strength would crystallization occur? So that, does urine provide an optimal condition for melamine crystallization?

Since melamine and cyanuric acid is held by hydrogen bonding, the theoretical ratio of melamine and cyanuric acid for melamine cyanurate crystallization is 1:1 but this need to be confirmed by an accurate and reliable crystallization method such as a mixed suspension, mixed product removal (MSMPR) system.

Urinary factors such as supersaturation and pH, are important factors that may affect melamine crystallization [161, 162]. This information would be vital as melamine is still allowable in foods and meats for human consumption according to the United States Food and Drug Administration (FDA). Furthermore, wide use of melamine in fertilizers is a crystallization risk factor which would potentially form renal stones.

1.6.7 Melamine crystallization with other lithogenic urinary factors

Calcium, oxalate and phosphate are commonly occurring lithogenic ions in urine.. Infants having melamine stones may at the same time have supersaturated calcium, oxalate, and/or phosphate lithogenic ions. However, the interaction between melamine and others lithogenic salts is unknown.

Structurally, melamine and cyanurate share very good epitaxial relationship with the endogenous uric acid (UA) found in human urine. This suggests that given the correct pH and supersaturation, UA may interact with melamine crystallization by co-precipitating with melamine so that the melamine cyanurate crystallization was inhibited. Cyanuric acid is an inhibitor of hepatic uric acid oxidase that leads to the enhancement of serum UA levels [155]. Excessive UA excreted in the renal tubules can compete with cyanuric acid for melamine binding. The clinicians did observe that UA was co-precipitated with melamine in infants and young children [163].

Urinary tract infection (UTI) is one of the most common bacterial infections in children [164]. At least 8% of girls and 2% of boys suffered from UTI in childhood [165]. Since melamine stones occurred in infants and child, UTI may contribute to melamine crystallization. *Escherichia coli* (*E. coli*) is the main bacterium (about 75% of UTI) isolate from urine [166]. The effect of *E. coli* on melamine crystallization is thus necessary to be investigated.

1.6.8 Melamine response to therapeutic agents

Potassium citrate and sodium bicarbonate are used clinically in the treatment of kidney stones. Urinary citrate forms a soluble complex with calcium by chelation that inhibits the formation and propagation of calcium-containing crystals [167] while bicarbonate enhances the urinary pH [168] to treat uric acid stones (alkaline treatment). These two therapeutic agents were also administrated to children with melamine stones. Therefore, the investigation of citrate and bicarbonate on the melamine crystallization can also suggest the usefulness of current therapy protocol to children.

1.6.9 Traditional Chinese medicines (TCM) on melamine crystallization

Traditional Chinese medicines (TCM) have been used for treating renal stones due to their anti-lithogenic activities. Previous study found that a Chinese herb Shi Wei (Folium Pyrrosiae) has a potential to inhibit urinary crystallization by reducing the urinary specific gravity and enhancing the urinary magnesium. Reducing the specific gravity of urine implied the reduction of urinary supersaturation and enhancing the urinary magnesium, which is an inhibitor of crystallization, that can complete with calcium to form ion complexes with oxalate (MgOx) which is more soluble than CaOx. Thus, this study aims to understand the effects of Shi Wei on melamine crystallization to see whether Shi Wei may also be a suitable therapeutic

agent for the prevention of the occurrence and recurrence of melamine stones in infants.

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Chapter 2: Approach and objectives

2.1 Approach to this study

This study investigates the urinary factors affecting calcium oxalate (CaOx) crystallization. There are many crystallization modifiers and two major classes were studied – urinary glycoproteins and urinary glycosaminoglycans (GAGs) by the mixed suspension mixed product removal (MSMPR) system which mimics the dynamic flow of urine in kidney.

GAGs were usually reported as inhibitors in CaOx crystallization while hyaluronan (HA) is reported as a promoter in CaOx crystallization which is distant from other GAGs species. Thus, both total urinary GAGs and HA excretion in stone-formers (SF), post-treated SF and compared with normal individuals were studied and correlated them with the occurrence and recurrence of CaOx stone diseases.

CD44 is known as a HA receptor and CD44 is found on various cells surface including epithelial cells and shown to bind with HA when released by cells in several studies due to cell injury induced by inflammatory response. Therefore, HA excretion and CD44 expression on renal cells in response to inflammatory response and also HA expression on CaOx induced cell injury were studied and suggested as possible markers during the subclinical inflammatory response induced by CaOx crystallization.

Melamine renal stone disease episode in China (2008) lead to the investigation on how melamine affected renal stone disease. Unlike CaOx crystallization, the physicochemical nature of melamine in urine and its handling were largely unknown at that time. Therefore, this was a new challenge to study the melamine crystallization based on the previously established MSMPR system. First of all, the physiochemical properties of melamine crystallization including pH and ionic strength were studied in order to give a clear idea if urine as a solvent provided an optimal condition for melamine crystallization.

To better understand the nature of melamine-induced renal stone disease, factors such as lithogenic salts including calcium, oxalate, uric acid, phosphate were studied. In addition, the risk of urinary tract infection (UTI) on melamine crystallization was also assessed as UTI is common in children. Currently used therapeutic agents on CaOx stone disease were also prescribed on melamine stone subjects. However, the effectiveness of these drugs on melamine crystallization was still unknown. Therefore, the effects of currently prescribed therapeutic agents including potassium citrate and sodium bicarbonate were investigated on melamine crystallization.

Traditional Chinese medicines (TCM) have been used for treating renal stones and is popular in China. Previous study found that a Chinese herb Shi Wei (Folium Pyrrosiae) has a potential to inhibit urinary crystallization by reducing the urinary specific gravity and enhancing the urinary magnesium. Thus, this study aims to understand the effects of Shi Wei on melamine crystallization to see whether Shi Wei

may also be a suitable therapeutic agent for the prevention of the occurrence and recurrence of melamine stones in infants.

2.2 Objectives

1. To measure the effects of total urinary glycosaminoglycans (GAGs), glycoproteins and individual GAGs on the calcium oxalate (CaOx) crystallization kinetics including the nucleation rates, growth rates and suspension densities by the mixed suspension mixed product removal (MSMPR) system that mimics the kidney with fresh supersaturated urine being continuously formed and passed through the kidney

2. To measure the excretion of urinary GAGs (protective) and hyaluronan (HA) (risk factor) in stone-formers (SF) (occurrence), post-treated (post-ESWL) SF (recurrence), and normal controls to reveal their relationship with renal stone disease

3. To measure the HA excretion and CD44 expression in the human kidney proximal epithelial (HK-2) cells under induced inflammatory conditions with inflammatory cytokine IL-1β

4. To measure the HA excretion in a two-compartment culture system with intact HK-2 cells monolayer under CaOx induced cell injury

5. To investigate the physiochemical properties (target at urinary pH and ionic strength) of melamine crystallization by MSMPR system

6. To study the interaction of melamine with other lithogenic salts including CaOx, uric acid and calcium phosphate by MSMPR system

7. To study the effects of urinary tract infection on melamine crystallization by addition of *E. coli* to the MSMPR system

8. To study the effectiveness of currently used therapeutic interventions (potassium citrate and sodium bicarbonate) for reduction of melamine crystallization by MSMPR system

9. To study melamine and cyanuric acid crystallization in MSMPR system with normal and male human urine

10. To study the effects of subject's human urine after Shi Wei supplementation on melamine crystallization by MSMPR system

Chapter 3

Urinary glycosaminoglycans and glycoproteins in a calcium oxalate crystallization system

3.1 Abstract

Background: This study measures the effects of total urinary glycosaminoglycans (GAGs), glycoproteins (GPs) and individual GAGs on the nucleation rates (Bo), growth rates (G) and suspension densities (M_T) of calcium oxalate (CaOx) crystallization by the mixed suspension mixed product removal (MSMPR) system.

Methods: Total urinary GAGs, GPs and individual GAGs including heparan sulphate (HS), chondroitin sulphate (CS) and Hyaluronic acid (HA) were added into the artificial urine (AU) and then introduced into the MSMPR test chamber and the crystal sizes and numbers were analyzed by a particle counter. The effects of added GAGs and GPs on CaOx crystallization were reflected by the changes on the crystallization indexes including the Bo, G and M_T of CaOx that were calculated based on the crystal size and numbers.

Results: Total urinary GAGs showed no statistical significance on both the Bo and G of the CaOx crystallization. However, both CS and HA enhanced Bo and suppressed the G when measured individually. CS enhanced the M_T while HA shown no significant change in the M_T of CaOx. HS enhanced the Bo as well as the M_T of CaOx crystals. Total urinary GPs showed an increase in the G and M_T of

crystals.

Conclusions: Although total urinary GAGs showed no statistically significant effect on CaOx crystallization, individual GAG showed that CS and HS promoted the CaOx crystallization while HA showed no significance in the crystal density of CaOx formed in the urine though it promoted the formation of smaller CaOx crystals. Total urinary GPs promoted the CaOx crystallization.

3.2 Introduction

Urolithiasis is the presence of renal stone and it is a common disease that is associated with high morbidity and recurrence rate due to the repeated stone episodes which leads to the economic burden [1] of urolithiasis. A retrospective study on idiopathic calcium urolithiasis showed recurrence rates of 14% at 1 year, 35% at 5 years, and 52% at 10 years [2]. Urolithiasis accounts for approximately 200,000 hospitalization per year in United States [2] and around 14,000 hospitalization in Hong Kong at 2009 [3].

The formation of renal stones is a consequence of increased urinary supersaturation with subsequent formation of crystalline particles [4]. Renal stones consist of about 98% crystalline materials [5] that are mainly of calcium salts including calcium oxalate (CaOx) and calcium phosphate [6]. Among the calculi, CaOx is by far the most common constituent of upper urinary tract calculi and also important in endemic bladder calculi as well [2] due to its low solubility and the high urine concentrations of calcium and oxalate normally excreted [7]. Stone formers tend to excrete urines that are more supersaturated than those of non-stone formers [4].

Renal stones do not consist of crystals alone as biomineralization requires an organic material called “matrix” for which the mineral is to be deposited on [8]. Therefore, stone matrix can be considered as the ‘framework’ of the stone. The stone matrix contains the organic material that may be derived from substances normally present in urine or may be produced by epithelial cells in the urinary tract

subsequent to the trauma induced by an enlarging stone [4]. A variety of urinary macromolecules (UMMs) may also act as the matrix macromolecules that regulate the crystal growth and nucleation.

Glycosaminoglycans (GAGs) are one of the major classes of UMMs that play an important role on the CaOx crystallization. They are a group of polysaccharides with repeated disaccharides units, in which one of the sugars is either N-acetylgalactosamine or N-acetylglucosamine or one of their derivatives [9]. GAGs are highly negatively charged because of the presence of carboxyl or sulphate groups on their sugar residues that lead to the stretched or extended conformation of polysaccharide chains of GAGs.

The majority of the GAGs in the urine appear to be derived from shedding from the urinary tract instead of glomerular filtration. Some studies showed that the urinary excretion of GAGs was significantly lower in stone formers than in normal individuals [10-14] while other studies demonstrated no significant differences between them [15-17]. There are five types of GAGs that are known to exist in urine including heparan sulphates (HS), dermatan sulphates (DS), chondroitin sulphates (CS), hyaluronic acid (HA) and in trace amounts keratan sulphate (KS). GAGs have also been isolated from the matrix of urinary stones [18, 19]. These studies showed that HA was found in apatite and struvite stones, HS in calcium oxalate monohydrate and uric acid stones and HA and HS in calcium oxalate dihydrate stones.

The increase production of GAGs by tubular epithelial cells may protect cells from toxic effects of calcium oxalate crystals and oxalate ions [20]. However, the role of GAGs on CaOx crystallization is still controversial since both inhibitory and promotory effects on crystallization were reported in many different studies [21-26].

The effects of urinary glycoproteins (GPs) on the CaOx crystallization remains unclear since controversial results were found in different studies. Tamm-Horsfall protein (THP) is the most abundant protein in normal human urine [27]. However, it is absent in CaOx crystals and only trace amounts of THP was found in the stone matrix [28]. A study demonstrated the promotory effect of THP on CaOx crystallization by enhancing the precipitation of CaOx [29] in concentrated urine, but another study showed no effect on spontaneous precipitation [30] and growth of CaOx crystals in vitro. Another study showed inhibitory effect of THP on CaOx crystal aggregation while no effect on the nucleation and growth of crystals [31]. Other urinary GPs such as inter-α-trypsin inhibitor (ITI), osetopontin (OPN), urinary prothrombin fragment-1 (UPTF-1), calgranulin and albumin were also reported with their inhibitory effects on the CaOx crystallization [32]. However, there is no study concerning about the effect of total urinary GPs on CaOx crystallization.

Many methods were developed for the studying of the CaOx crystallization in vitro. In general, the supersaturation will decrease when crystallization occurs since calcium and oxalate were removed form the solution and thus the kinetics of the crystallization process will be affected unless the supersaturation is maintained by continuous refill of the materials for the test. A mixed suspension mixed product

removal (MSMPR) system was applied in this study since it is a continuous flow system with fresh supersaturated artificial urine (AU) being continuously flowing through the crystallizer that can mimic the kidney with fresh supersaturated urine being continuously formed and passed through the kidney and thus a steady state supersaturation being achieved. AU with addition of GAGs or GPs can be added to a test chamber and a parallel chamber with AU added is used as the control chamber of the MSMPR system so that the changes in the growth rate (G), nucleation rate (Bo) and suspension density (M_T) of the CaOx crystals can be compared at the same time independently. Since the milieu of urine is complex, important modifiers of urinary calcium oxalate in AU were investigated before studying in whole urine in subsequent study.

3.3 Materials and methods

3.3.1 Urine collection

24 hours urine form each of 5 healthy individuals without history of kidney stone disease were collected into 3 liters bottles without preservative. The urine samples were refrigerated at 4℃ during 24 hours collection. Urinalysis was performed on all samples to confirm that the subjects were free from urinary tract infection and bacteria contamination. Then, 550 mL of urine samples from each individual, after centrifugation, were pooled for the recovery of the total urinary GPs and GAGs.

3.3.2 Recovery of total urinary GPs

Total urinary GPs were recovered by the ammonium sulfate protein precipitation method. Briefly, 1.767 kg of ammonium sulfate was added slowly to 2.5 L of pooled urine with continuous stirring to create a saturated ammonium sulfate solution for protein precipitation overnight at 4℃. The proteins were recovered after centrifugation and then dissolved in 1 mL of 1 M of ammonium sulfate solution for dialysis with 1 L of AU through a 10 kDa cut-off Spectra Biotech Cellulose Ester (CE) dialysis membrane (Spectrum Laboratory Inc., Rancho Dominguez, CA). Several changes of AU were done during the 72 hours dialysis period to remove excessive salts.

3.3.3 Recovery of urinary total GAGs

Urinary polyanionic macromolecules (UPMs) which were recovered by sequential cetylpyridinium chloride (CPC) and then sodium acetate-saturated ethanol (EtOH) precipitation as described previously [33]. Briefly, 250 mL of pooled urine was diluted with 3 volumes of 0.025 M sodium acetate buffer at pH 5.8. Then, 0.1 volumes of 5% CPC in acetate buffer were added into the buffered urine. The precipitate was washed with cool distilled water and then dissolved in propanol. 4 volumes of EtOH were added to precipitate the UPMs as sodium salts. Total urinary GAGs were recovered by the papain digestion of the precipitate followed by another sequential CPC-EtOH precipitation.

3.3.4 Individual GAGs

Three types of GAGs including HS derived from bovine kidney, chondroitin sulphate-A (CS-A) derived from whale cartilage and HA derived from pig skin were obtained commercially from Seikagaku Corporation, Tokyo, Japan.

3.3.5 Preparation of AU

The constituents of the AU (Table 3.1) used in this study were as described in Kavanagh's study [7]. All chemicals were of analytical grade. The chemicals of AU were dissolved in MilliQ water and then the pH was buffered to 6.0 with hydrochloric acid, using phosphate as the buffering ion.

Table 3.1. The constituents of artificial urine (AU). (Kavanagh *et al* 1999)

Chemical Formulae	M.W.	Concentration (mmol/L)	Mass for 1L AU (g)
NaCl	58.44	160	9.35
KCl	74.55	82.1	6.12
Na_2HPO_4	179.99	25.0	4.45
$(NH_4)_2SO_4$	132.14	21.9	2.89
NH_4Cl	53.49	3.5	0.19
$MgCl_2$	95.22	3.26	0.31
$K_3C_6H_5O_7$	324.408	2.17	0.70

3.3.6 Establishment of mixed suspension mixed product removal (MSMPR) system

A pair of identical MSMPR crystallizers (20 mL reaction volume, thermostated at 37℃ with continuous stirred) in parallel (test and control chambers) had been developed for crystallization studies [34]. A close fitting lid covered the opening of the crystallizer to minimize evaporation and with 4 openings for the fitting of inlets of AU, 150 mmol/L of calcium chloride (CaCl) solution, 30 mmol/L of sodium oxalate (NaOx) solution, and outlet tubes for mixed product removal.

A flow rate of 2.64 mL/minute for the AU and 0.11 mL/minute for the CaCl and NaOx solutions were applied so that the flow rate at the outlets of the crystallizer was the sum of the three feed solutions that is 2.86 mL/minute with the proportion of 92% AU and two 4% feed solutions of CaCl and NaOx. The whole set up of MSMPR system is shown in Figure 3.1.

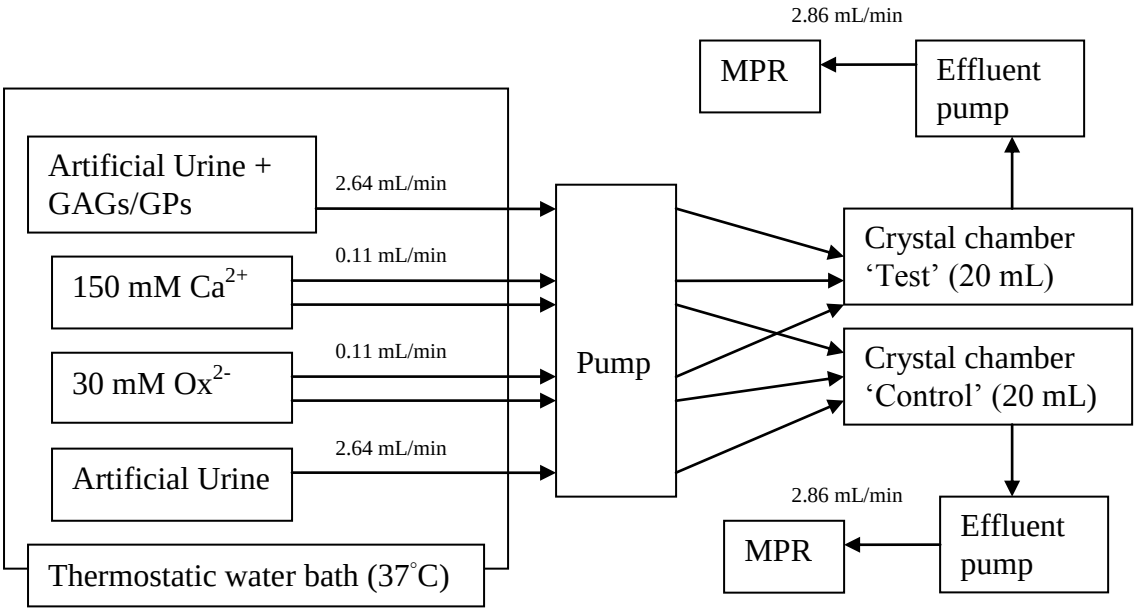


Figure 3.1. Schematic diagram of the MSMPR crystallizer. Artificial urine, calcium and oxalate ions were added into both test and control chambers from three inlet tubes. Glycosaminoglycans (GAGs) or total glycoproteins (GPs) for testing were added into the test artificial urine. An outlet tube from each chamber was for mixed product removal for crystal size and number measurement by a particle counter. The flow rate at the outlets of the crystallizer was the sum of the three feed solutions that is 2.86 mL/minute to keep the equilibrium volume of urine inside the chamber throughout the run.

3.3.7 Comparison of the similarity between the control chamber and test chamber

The control and test chambers were optimized to give similar crystallization properties before the start of experiments. The three feed solutions were equilibrated to 37℃ before the experiments were begun. Then the two chambers were run simultaneously with the AU, CaCl and NaOx. The two chambers were allowed to run for 7 to 8 residence times [7, 24] where 1 residence time is equal to (20 mL ÷ 2.86 mL/minute = 7 minute) that is about 50 minutes for equilibration of the MSMPR system. After that, 10 consecutive measurements of crystal numbers and sizes were obtained with 7 minutes intervals for each chamber by using the Coulter Multisizer 3 (Beckman Coulter, USA) particle size analyzer with 300 channels. All tests were performed in triplicate. The crystal number and size were found to remain constant after 8 residence times. The parameters of growth rate (G, μm/minute), nucleation rate (Bo, numbers/minute/mL) and suspension density (M_T, mmol/L or mM) were determined from the number and size of crystals as previously described [7, 24] in chapter 1.

The calcium oxalate crystallization parameters including the G, Bo and M_T of crystals were calculated and the differences between the medians between the test and control chambers were shown to be statistically insignificant when compared by the Mann-Whitney test. Therefore, the 2 chambers were considered to be of similar crystallization properties.

3.3.8 Comparison of control chamber with the test chamber with addition of GAG or GPs in AU

The procedures were based as previously described except one type of GAG or total GAGs or GPs in AU was added to the test chamber for each run. After equilibration of the system, 10 consecutive measurements of crystal particle numbers and sizes were obtained with 7 minutes intervals for each chamber. The parameters of G, Bo and M_T were then calculated as previously published [7, 24, 35].

3.3.9 Comparison of different concentrations of GAGs and GPs on the effects of CaOx crystallization

Based on the daily urinary excretion of total GAGs which is around 25 µg per day [16] in about 2 Liters of urine, 25 µg of GAGs are present and around 12.5 µg/L of GAGs in AU is established for this experiment. 5, 12.5 and 25 µg/L of different types of GAGs and same concentrations of GPs in AU were prepared for the experiment. 10 runs for each concentration of GAGs and GPs were obtained and the crystallization parameters of G and Bo as well as M_T were calculated based on the crystal particle numbers and sizes recorded.

3.3.10 Statistical analysis

GraphPad Prism version 4.03 for window (GraphPad Software, San Diego California, USA) was used to perform all statistical analysis in this experiment. The slope and y-intercept of the plot of Ln (N) against crystal sizes was tested by linear regression. All reported findings were with a linear plot with a high r^2 (>0.95) represented a realistic growth and nucleation rates without effects of the aggregation of crystals. One-way analysis of variance (ANOVA) was used for the comparison of the mean differences between different concentrations of GAGs or GPs and control. A *P* value of less than 0.05 was considered to be statistically significant. All significant ANOVA test results were analysis by Dunnett's multiple comparisons post-test. The *P* value of less than 0.05, 0.01 and 0.001 were specified by the symbols *, **, and *** respectively in graphs and table 1.

3.4 Results

3.4.1 Effects of GAGs on CaOx crystallization

3.4.1.1 Effect of total urinary GAGs

Total urinary GAGs showed no statistical significance on both the nucleation (Bo) (Figure 3.2B) and growth rates (G) (Figure 3.2A) of the CaOx crystallization. There was a significance increase in the suspension density (M_T) of CaOx crystals at 5 μg/L of total GAGs only but no increasing trend of M_T was observed by increasing total GAGs concentrations.

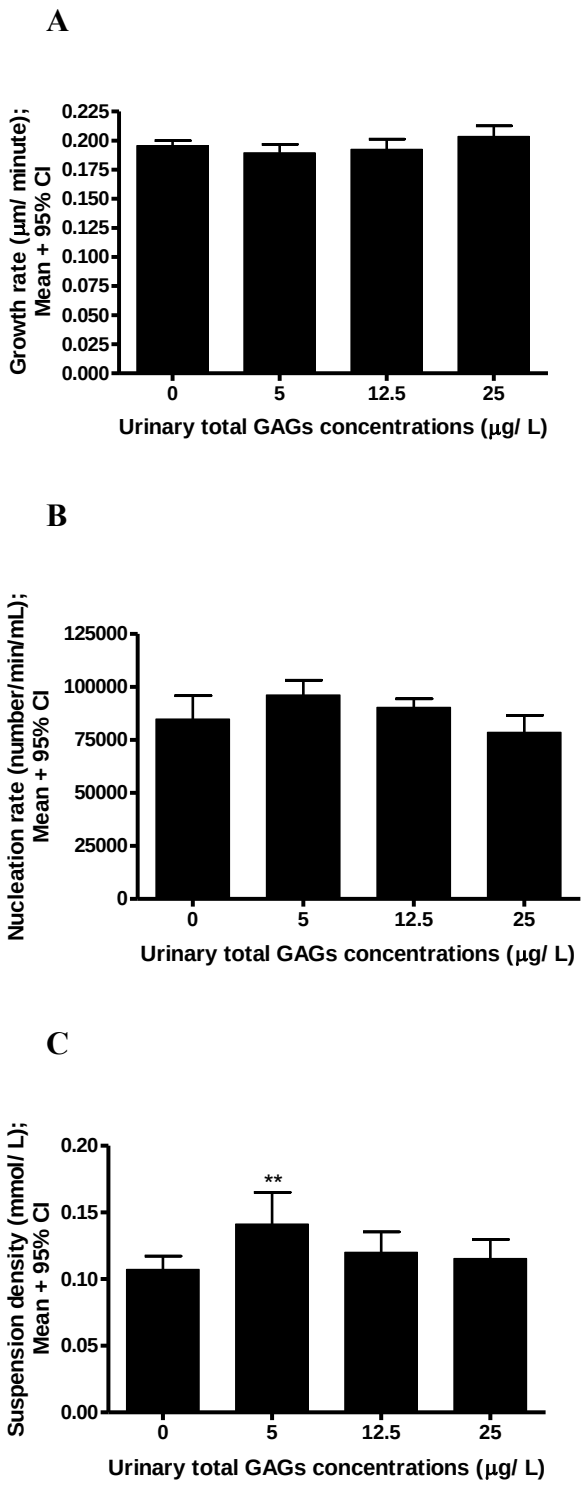


Figure 3.2. The effects of total urinary GAGs on the (A) growth rates, (B) nucleation rates and (C) suspension densities (mean + 95% CI) of CaOx crystals.

3.4.2 Effect of individual GAGs

3.4.2.1 Effect of HS

HS enhanced the Bo (Figure 3.3B) as well as the Mᴛ (Figure 3.3C) of CaOx crystals. This suggested that HS promoted the CaOx crystallization by increasing in the number of CaOx crystals. No trend of increasing concentrations of HS on the G of CaOx crystals since there was a significance reduction in the G by adding 12.5µg/L of HS but a significance increase in the G by adding 25µg/L of HS.

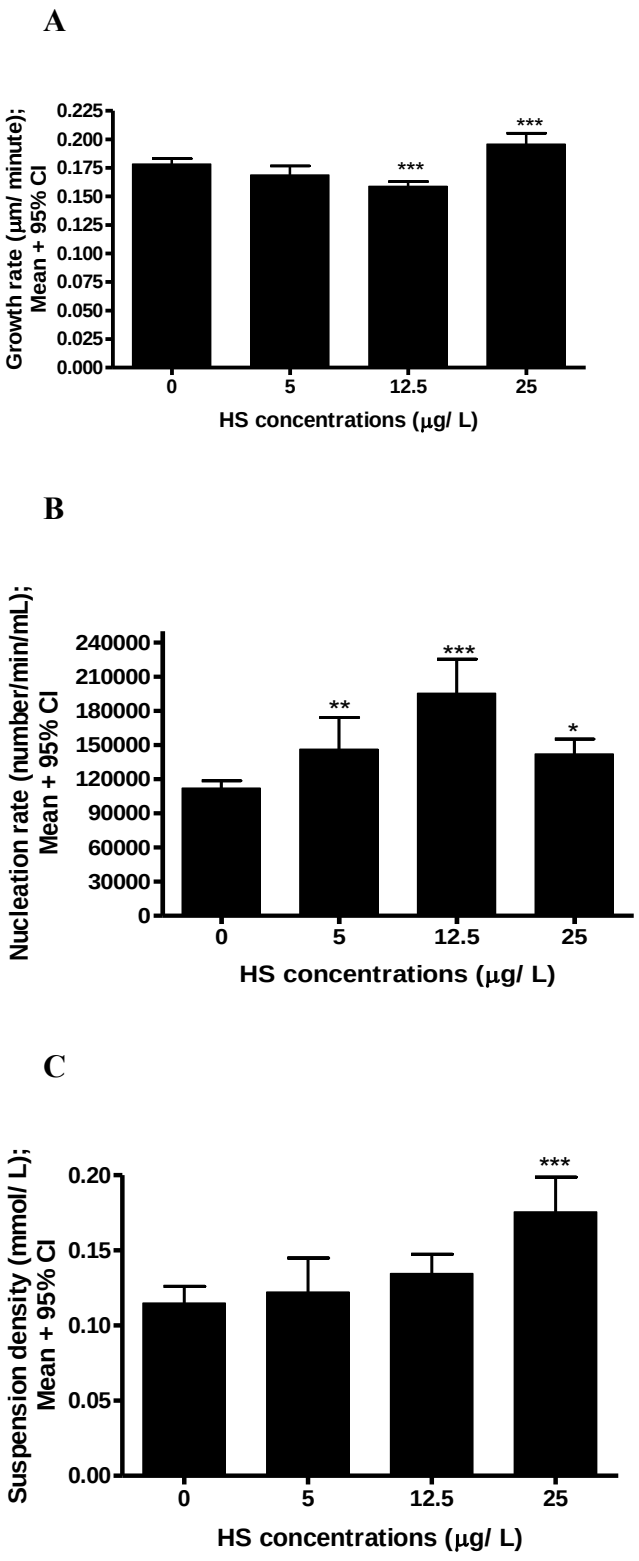


Figure 3.3. The effect of HS on the (A) growth rates, (B) nucleation rates and (C)

suspension densities (mean + 95% CI) of CaOx crystals.

3.4.2.2 Effect of CS and HA

CS and HA enhanced the Bo (Figure 3.4B and 3.5B) and suppressed the G (Figure 3.4A and 3.5A) of the CaOx crystals. However, CS enhanced the MT (Figure 3.4C) while HA showed no significant change in the MT (Figure 3.5C) of CaOx. CS promoted the CaOx crystallization by means of producing more smaller CaOx crystals. HA showed no significant changes in the density of CaOx formed in the urine however it also promoted the formation of smaller CaOx crystals.

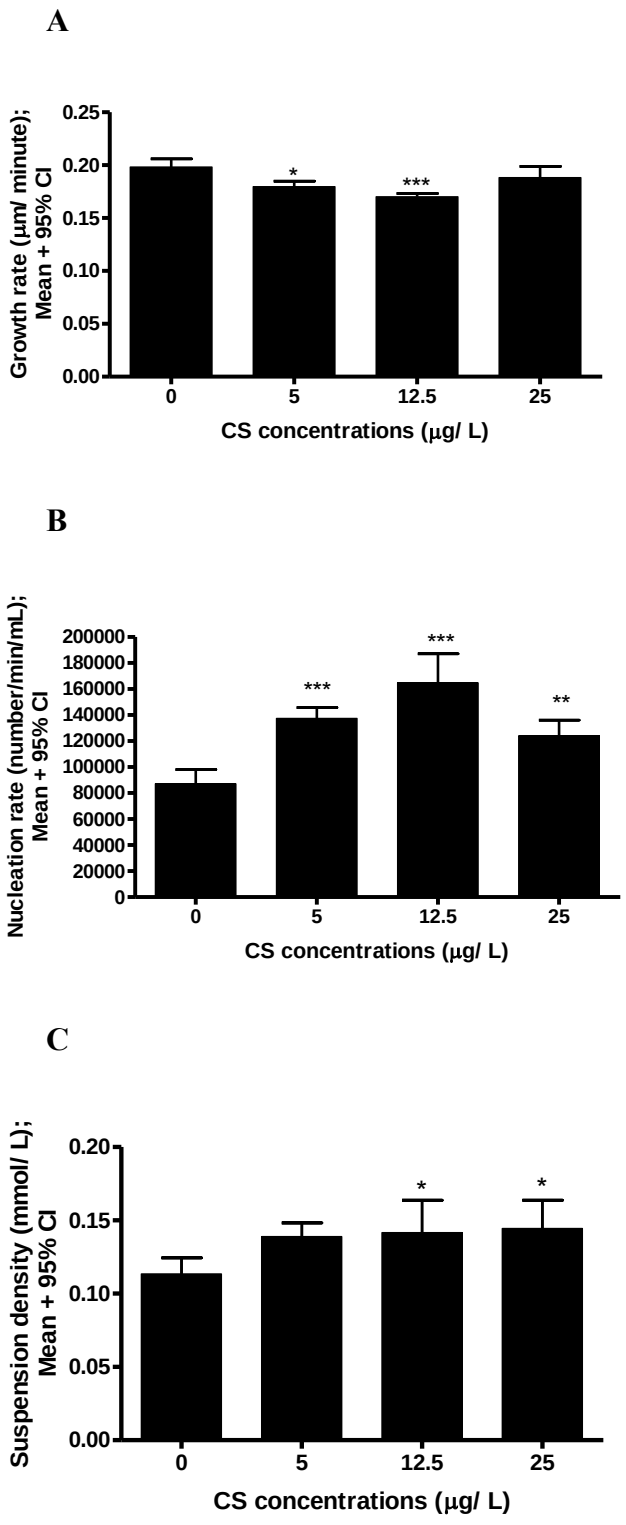


Figure 3.4. The effect of CS on the (A) growth rates, (B) nucleation rates and (C) suspension densities (mean + 95% CI) of CaOx crystals.

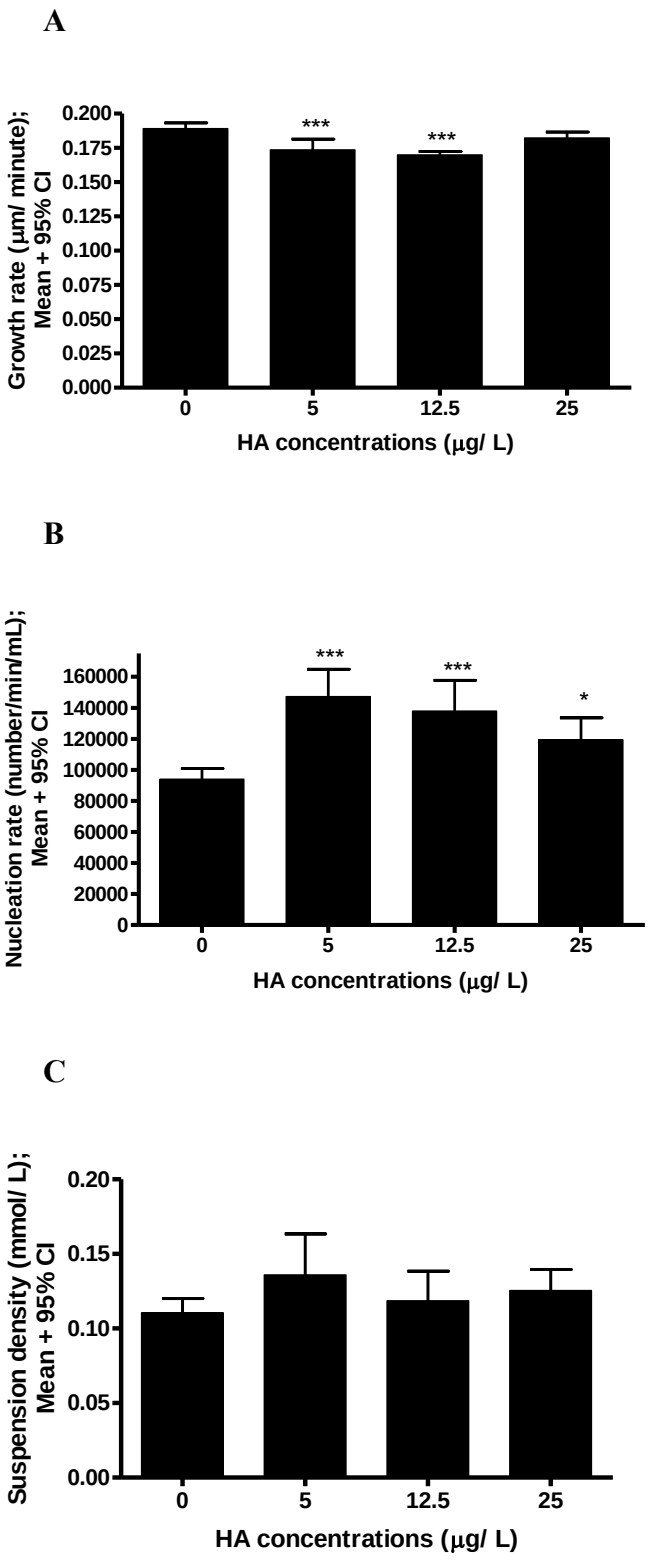


Figure 3.5. The effect of HA on the (A) growth rates, (B) nucleation rates and (C) suspension densities (mean + 95% CI) of CaOx crystals.

3.4.3 Effects of total urinary GPs on CaOx crystallization

Total urinary GPs showed a concentration-dependent increase in the G (Figure 3.6A) and Mᴛ (Figure 3.6C) of crystals. This indicated that total urinary GPs promoted CaOx crystallization by formation of larger crystals.

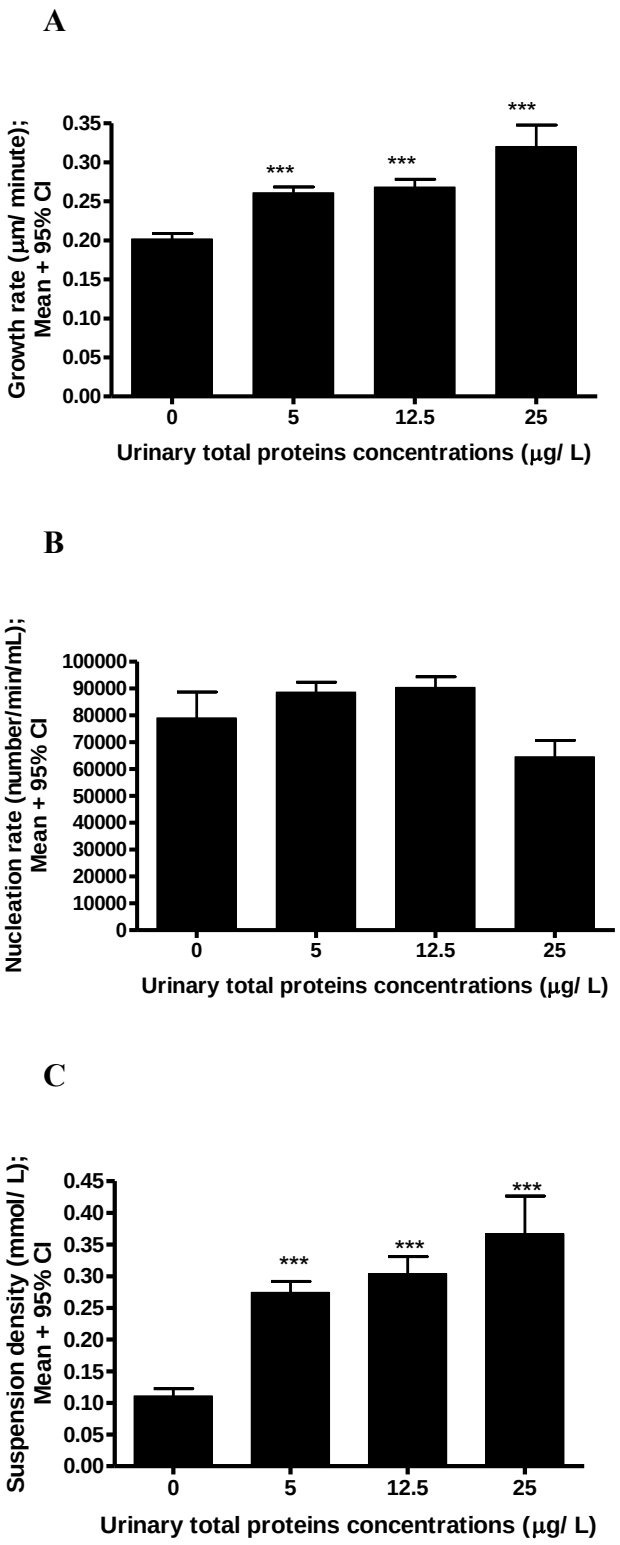
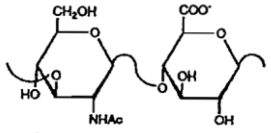
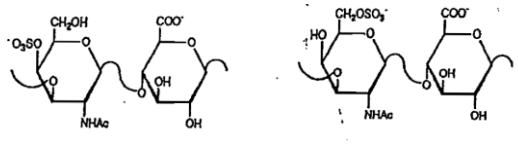
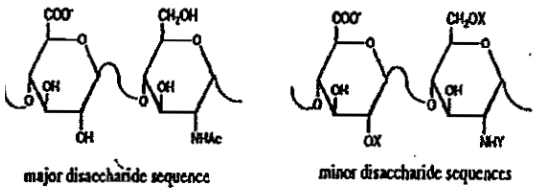


Figure 3.6. The effect of total urinary glycoproteins on the (A) growth rates, (B) nucleation rates and (C) suspension densities (mean + 95% CI) of CaOx crystals.

Table 3.2. Indicative trend of calcium oxalate crystallization indices from the MSMPR crystallizer for nucleation rate ($\mathbf{B_o}$; number/min./mL),growth rate (**G**; µm/min.) and suspension density ($\mathbf{M_T}$; mM) are indicated by arrows for any increases (↑), decreases (↓) and no-changes (↔).

	$\mathbf{B_o}$	**G**	$\mathbf{M_T}$
Total glycosaminoglycans	↔	↔	↔
Hyaluronic acid (HA)	↑***	↓***	↔
			
Chondroitin sulphate (CS)	↑***	↓***	↑*
			
Heparan sulphate (HS)	↑***	↓***	↑***
 major disaccharide sequence minor disaccharide sequences			
Total glycoproteins	↔	↑***	↑***

*P < 0.05; **P<0.01; ***P<0.001

3.5 Discussion

Previously, the urinary GAGs excreted (in terms of µg of hexuronate per mL of urine) from normal subjects and stone formers were determined to be in the range of 2-6 µg/mL of urine [22, 33]. In terms of total GAGs, minus the glycoproteins, this is equivalent to an excretion of 1 - 5 mg per day, however. This amount of daily excretion of GAGs in urine is sufficient to be involved in crystallization events for calcium oxalate as tested before [10, 33]. The concentration range for GAGs (in terms of dry weight) used in the crystallization studies is within this range of around 10 µg/L per 24-hr urine [16].

Total urinary GAGs and GPs are the main macromolecules that are regarded as modulators of the CaOx crystallization. They can enhance the urinary supersaturation by forming complex with calcium salts so that higher concentration of calcium can be tolerated in the urine without being precipitated out as CaOx crystals. However, the total urinary GAGs showed no statistical significance on both the Bo and G of the CaOx crystallization in this study (Figure 3.2). Therefore, it is suggested that the inhibitory effect of GAGs on CaOx crystallization is not due to its effects on the CaOx crystals size and amounts directly but it is more likely that the highly negatively charged urinary GAGs prevented the binding of CaOx crystals onto the negatively charged urinary epithelial cells surface by itself being covered on the surface of the crystals [36]. Moreover, the highly negatively charged GAGs leads to the stretched or extended conformation of polysaccharide chains [37] as an expanded random coil structure [38] due to the presence of carboxyl or

sulphate groups on sugar residues. Therefore, the crystals cannot bind to the surface of the urinary epithelial cells by both the charge repulsion and the hindrance by the extended conformation of GAGs and hence the crystals cannot be retained and grow. Studies have shown conclusively that the adherence of CaOx monohydrate (COM) crystals to renal epithelial cells such as Madin-Darby canine kidney (MDCK) [39, 40] and nontransformed African green monkey (BSC-1) cells [36], both were significantly inhibited by the addition of GAGs. GAGs inhibited the binding of COM crystals onto the BSC-1 cells by itself being coated onto the crystals instead of coating onto the cells [36] and similar results were shown with semi-synthetic polysaccharides (SSPs) that mimic the structure of GAGs which were potent inhibitors for the cell-crystals interaction on the COM binding of the MDCK cells [39] when the crystals were pretreated with SSPs but not the pretreated cells.

It is interesting that all individual GAGs have shown to alter CaOx crystallization activities in this study (Table 3.2). Amongst the 3 GAGs tested, HS is the major GAG that can be extracted from stone matrix material [18, 19, 41]. HS enhanced the Bo of CaOx crystals (Figure 3.3B) that is correlated with other studies and the material extracted from stones, presumably containing HS, had been shown to enhance the Bo [10] of CaOx crystals and inhibit crystal growth [10] in urine. It may be due to its chemical structure [42] such as its peptide content, length of the carbohydrate-peptide linkage region, degree of sulphation, or its molecular size that enhances its absorption onto crystals. However, HS increased the M_T of the CaOx crystals (Figure 3.3C) implicating that HS acted on the crystals by producing more smaller crystals.

CS is the major GAG present in human urine [23, 32]. The results showed a reduction in the G (Figure 3.4A) with an increase in the Bo (Figure 3.4B) of CaOx crystals by the addition of CS-A. This is contradictory to the studies that CS inhibits crystal Bo [23, 43] but promotes G of crystals. Since CS is absent in both the CaOx crystals and stone matrix [18, 19, 44], it is hypothesized that the action of CS in CaOx crystallization may be related to the interaction between CS to others modulators for CaOx crystallization rather than directly acting on the crystals or stones. CS also enhanced the MT of the CaOx crystals (Figure 3.4C) suggesting that CS resulted in the production of more smaller crystals.

HA is found in urine [22, 32] as well as in the stone matrix [18, 19]. It altered the CaOx crystallization by enhancing the Bo (Figure 3.5B) and reducing the G (Figure 3.5A) of crystals that also lead to produce smaller crystals. Urinary HA is excreted in a significantly higher proportion (11.6% of total GAGs) from stone-formers than HA in the urine of normal subjects (6.2%) [22]. It is suggested to be a consequence of active turnover of renal tissue in the diseased state during which HA fragments were released into urinary tract [45]. Studies showed that migrating cells produce large amounts of HA during repair of damaged renal epithelial cells [46, 47]. These suggested that HA produced during inflammation of renal epithelial cells may reduce the risk of renal stone formation by its inhibitory effect on the CaOx crystallization.

However, the HA produced is known to bind to its receptors CD-44 on the surface of epithelial kidney cells [48]. This causes the cell surface to become sticky

and enhance the crystal-cell binding in cell culture model [36, 49]. HA has been found to bind CaOx crystals at the surface of proliferating renal tubular cells in cell culture model. It acts as a crystal-binding molecule at the surface of MDCK [50] and BSC-1 [36] cells which are involved in the adherence of COM crystals, and considered as a critical event in the pathophysiology of calcium nephrolithiasis.

The mechanism by which GAGs influence the crystallization process is still unknown and it is worthy of investigation of the function of GAGs in the CaOx crystallization in terms of its structural difference between different kinds of GAGs.

Total urinary GPs did significantly enhance the CaOx crystallization (Figure 3.6) by producing more and larger crystals in a dose-dependent manner. Since we extracted total GPs from urine, the main GP [27] recovered was THP. Although THP is absent in CaOx crystals and only tract amounts of THP were found on the stone matrix [28], it may affect CaOx crystallization by interaction with other urinary lithogenic factors. Results from this study confirm with other study that demonstrated a promotory effect of THP on CaOx crystallization by enhancing the precipitation of CaOx in concentrated urine [29], but another study showed no effect on spontaneous precipitation and growth [30] of CaOx crystals in vitro. Another study showed inhibitory effect of THP on CaOx crystal aggregation while no effect on the nucleation and growth [31] of crystals. However, it is contradictory with another study that THP inhibits the growth of CaOx [51]. Other urinary proteins have also been reported with specific roles on the CaOx crystallization. Bikunin produced by kidney [52] and enhanced expression of bikunin mRNA was observed in renal epithelial cells exposed to oxalate and CaOx crystals [53, 54] and

it is a potent inhibitor of CaOx crystallization by inhibition of nucleation and growth of CaOx crystals [55]. Increased expression of osteopontin (OPN) was observed when renal tissue was damage by CaOx stones or crystals [56] and OPN is a potent inhibitor of nucleation [57], growth and aggregation [58] of CaOx crystals. Urinary albumin is abundant in urine [59, 60] and also detectable in the matrix of urinary stones [60] and crystals [61]. Studies have shown that albumin can bind to CaOx crystals [62, 63] but no inhibitory effect on CaOx growth [31, 62]. Other studies have shown the promotory effect of albumin on the crystal nucleation [64]. These contradictory results within and between each individual urinary GPs make the explanations of the actions of total urinary GPs on CaOx crystallization become more complex, and thus requires further elucidation.

It is therefore not surprising that even over the years, there has not been much progress or papers for elucidation of the role of GAGs and GPs in CaOx urolithiasis due to their heterogeneous structure and carbohydrate side-chains. Given the vast array of urinary proteins present in the urine, it is more than likely that these urinary proteins (and GAGs) work in some synergistic way as that proposed under the biomineralization model. For many instances, the roles of the urinary proteins and GAGs is very system–dependant. The crystallization systems vary in the use of artificial urine, undiluted, diluted or fractionated urine (ultrafiltrates) to show crystal activities. Here we have attempted to study the proteins and GAGs under one system – the MSMPR crystallizer.

It is interesting that total GPs had such a strong promotory effect on CaOx crystallization when compared to total GAGs since both of them are anionic, with

many acidic amino acid residues and with post-translational modifications such as sulphation, phosphorylation and glycosylation. Also, those urinary macromolecules (UMMs) usually considered as inhibitors on CaOx crystallization were found to be promoter of crystallization. Further studies are needed on the composition of GPs and also the expression and regulation of these GPs and their actions on the urinary environment as well as during the CaOx crystallization that may provide the clue for the prevention and treatment of CaOx stone disease.

3.6 Conclusion

Although total urinary GAGs showed no statistical significant effects on CaOx crystallization, individual GAGs showed that CS and HS promoted the CaOx crystallization while HA showed no significant changes in the density of CaOx formed in the urine but it promoted the formation of smaller CaOx crystals. This suggests a different mechanism of reducing supersaturation. Total urinary GPs promoted the CaOx crystallization, however, the explanations are complex due to its abundance, nature of amino acids, methods of study and nature of modifications present.

This study reveals possible mechanism by which individual GAGs, whole GAGs (as a polymer) and glycoproteins may influence crystallization in vivo whilst present in urine. Whole GAGs and glycoproteins, as a polymer or aggregate affects through their shape, form and overall charge amassing crystal aggregates with the risk of forming larger stones as evidence by M_T (larger sizes and suspension densities). Whilst the individual GAGs (all three) increase rate of nucleation and lower growth rate with increased suspension density by favoring smaller crystals which are easy to pass out reducing risk of forming stones. HA was an exception for M_T.

Further studies are needed (1) to identify other components of whole GAGs which ‘neutralizes’ them to affect the crystallization and (2) how to remove or suppress them.

3.7 References

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Chapter 4

Hyaluronan is an accidental risk factor for recurrent calcium oxalate stone formers

4.1 Abstract

Background: Glycosaminoglycans (GAGs) protect cells from binding of calcium oxalate (CaOx) crystals to prevent CaOx stone formation. Hyaluronan (HA) is a non-sulfated GAG with crystallization-promoting activity during renal stone formation. The excretion of urinary GAGs and HA were measured in stone-formers (SF), post-treated SF, and normal controls to reveal their relationship with renal stone disease.

Methods: Three groups were recruited – active SF, post-treated SF (SF after extracorporeal shock wave lithotripsy) and normal controls with 40 subjects in each group were recruited. Early morning urine was collected for each subject. The hexuronate content of the GAGs were measured by carbazole reaction and values for GAGs were standardized against creatinine. Individual GAGs extracts were then digested sequentially with *Streptomyces* hyaluronidase and chondroitinase ABC to yield the HA disaccharides for analysis by high performance liquid chromatography.

Results: Hexuronate content of GAGs were in the order of post-treated SF < SF << normal controls. SF had an enhanced urinary excretion of HA with no increase of

urinary HA in post-treated SF group. However, both of the SF and post-treated SF groups had increased proportion of HA (of total GAGs) compared to normal controls.

Conclusion: Active-SF and post-treated SF groups had lower total GAGs content but increased proportion of HA than that of the normal controls indicating that urinary GAGs and HA are probably protective/risk factors respectively for the renal stone disease. The higher recurrence rate of renal stone disease may be due to the sub-species of GAGs that may become an accidental participant for renal stone formation.

4.2 Introduction

Increased urinary supersaturation with subsequent formation of crystalline particles that retains, leads to the formation of renal stones [1]. The most common type is calcium oxalate (CaOx) stones [2, 3]. Normally, most of the solid particles crystallizing within the urinary tract will be excreted freely in urine since an intact and functional epithelium formed by renal tubular cells is non-adherent to crystals [4, 5]. However, when crystals are retained by the injured renal epithelial [4], they can develop to full-size stones [4, 6]. The increased production of glycosaminoglycans (GAGs) by tubular epithelial cells protect cells from binding of crystals [7] that lead to stone formation. Some studies have shown that the urinary excretion of GAGs was significantly lower in stone formers (SF) than in normal individuals [8-10] while other studies demonstrated no significant differences between them [11, 12]. However, there are no systematic studies concerning urinary GAG excretion and sub-GAG species in stone-formers after stone removal by extracorporeal shock wave lithotripsy (post-ESWL) that may affect the relative risk for the recurrence of renal stones.

Hyaluronan (HA) is a non-sulfated GAG with crystallization-promoting properties [13, 14] and up-regulation of HA was observed in human kidney proximal epithelial (HK-2) cells during CaOx crystals induced cell injury [15] suggesting HA as a mediator for repairing of an injured epithelium. Urinary HA is excreted in a higher proportion from stone-formers than HA found in the urine of normal subjects [13]. Thus, an increase in urinary HA excretion can indicate renal cell injury suggesting a risk for formation of renal stones. In this study, the urinary

GAGs and HA measured from active stone-formers (SF), post-treated SF, and normal individuals were investigated in order to find out their relationship with renal stone disease.

4.3 Materials and Methods

4.3.1 Subject recruitments and collection of urine samples

Three groups of subjects including both male and female aged from 18 to 65 (n = 40 for each group) were recruited. These including the "active SF" – confirmed with the presence of renal stones by radiographic examination and "post-treated SF" which were SF after extracorporeal shock wave lithotripsy (post-ESWL) and declared "stone-free" by radiological and ultrasound assessments. Kidney functions of post-treated SF were determined to be normal after 2 weeks at the follow-up examination at hospital. These two groups were recruited from the Lithotripsy unit, Department of Surgery, Queen Mary Hospital, Hong Kong. Aged-matched normal subjects group with no known urological history were randomly recruited from Hong Kong community as controls. All subjects had given informed consent to participant in this study. Ethics approval was obtained from the Human Subjects Ethics Sub-committee of the Hong Kong Polytechnic University and from the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster.

All subjects were confirmed without diseases (e.g. diabetics mellitus or hypertension with medications) that may affect the renal functions. Early morning urine (EMU) were collected for each subject after recruitment except subjects from post-ESWL group that were collected at 14 days after treatment. Urinalysis were performed on all samples to confirm that they were without evidence of urinary

tract infection and confirm the integrity (e.g. no protein, red blood cell, glucose, etc.) of the urine specimens.

4.3.2 Recovery of urinary GAGs

Urinary GAGs were recovered by a protocol previously established [13]. Briefly, EMU was diluted with 3 volumes of 0.025 M sodium acetate buffer at pH 5.8. Then, 0.1 volume of 5% cetylpyridinium chloride (CPC) in acetate buffer was added into the buffered urine. The precipitate was washed with cool distilled water and then dissolved in propanol. 4 volumes of sodium acetate-saturated ethanol (EtOH) were added to precipitate the urinary polyanionic macromolecules as sodium salts. Urinary GAGs were recovered by the papain (Sigma Chemical Co., St. Louis, USA) digestion of the precipitate at 65℃ followed by another sequential CPC-EtOH precipitation.

4.3.3 Analysis of the hexuronate content of urinary GAGs

The hexuronate content of the GAGs were detected from a glucuronolactone standard curve that were measured by carbazole reaction with reference to Bitter & Muir [16] and Gohel & Shum [14]. All samples were done in triplicate. The results of hexuronate in GAGs were standardized against creatinine.

4.3.4 Urinary creatinine determination

Pre-diluted urine samples (1:50) with MilliQ water were analyzed by the automated chemistry analyzer (Cobas Fara, Roche Diagnostics, USA) with the creatinine regents (BioSystems, Barcelona, Spain) according to the manufacturer's specifications. All samples were performed in triplicate.

4.3.5 Double enzyme digestions of GAGs for HA disaccharides

Individual GAGs extracts and HA from Pig skin (Seikagaku Corporation, Tokyo, Japan) for HA standards were digested sequentially with 0.3 turbidity reducing units of hyaluronidase from *Streptomyces hyalurolyticus* (Seikagaku Corporation, Tokyo, Japan) and 0.1 U of chondroitinase ABC from *Proteus Vulgaris* (Seikagaku Corporation, Tokyo, Japan) as previously described [13] to yield the HA disaccharides.

4.3.6 High performance liquid chromatography (HPLC) analysis of HA disaccharides

HA disaccharides quantitation was performed by a Waters HPLC system (Waters Corporation, Milford, USA) comprising of a Waters Alliance separation module 2695, a Waters photodiode array detector 2996, and an Empower Pro software (Waters Corporation, Milford, USA). The settings on the HPLC system was based on the previous protocols [13] with modifications. Double enzymes digested samples and HA standards as well as Di-HA standard for HPLC from a

unsaturated GAG disaccharide kit [ΔDermato/Hyaluro-Disaccharide Kit (D-Kit) from Seikagaku Corporation, Tokyo, Japan] were reconstituted with mobile phase and injected into a 250 x 4.6 mm Econosphere NH_2 5µm column (Grace, Deerfield, USA) connected with a 7.5 x 4.6 mm Econosphere NH_2 5µm Alltech guard column (Grace, Deerfield, USA) with 5mM sodium dihydrogen orthophosphate/ orthrophosphoric acid (pH 2.60) as mobile phase at a flow rate of 1 mL/minute. The column eluate was monitored at 232 nm and the peak area were determined. The retention time for HA disaccharides of processed urine samples (Figure 4.1A) and HA standards (Figure 4.1B) were determined at 7.5 minutes with reference to Di-HA HPLC standards (Figure 4.1C). HA disaccharides in samples were detected from HA standard curve and standardized against creatinine.

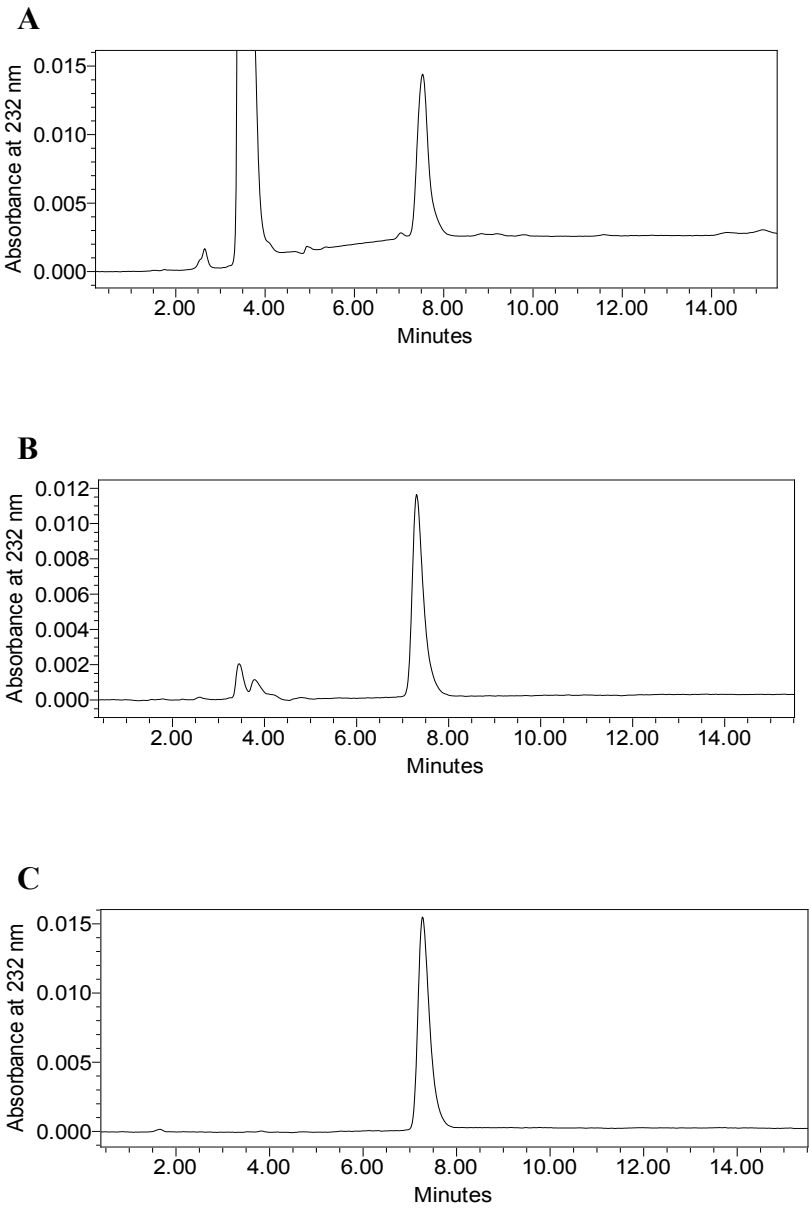

Figure 4.1. Chromatograms of hyaluronan (HA) disaccharides after sequential *Streptomyces* hyaluronase and chondroitinase ABC digestions of urinary glycosaminoglycans of (A) urine sample, (B) 1.25 μg HA standard. Retention time for HA disaccharides were determined at 7.5 minutes with reference to (C) 1.25 μg Di-HA HPLC standard.

4.3.7 Statistical analysis

GraphPad Prism version 4.03 for window (GraphPad Software, San Diego California, USA) was used to perform all statistical analysis in this experiment. One-way analysis of variance (ANOVA) was used for the comparison of the mean differences of GAGs and HA between SF, Post-SF and normal individuals groups. Results were reported as mean $\pm$ 95% confidence interval (C.I.). A *P* value of less than 0.05 was considered a significant difference and specified by the symbol * in the graphs. All significant ANOVA test results were analysis by Tukey's multiple comparison post-test.

4.4 Results

4.4.1 Total glycosaminoglycans (GAGs) excretion [(µg hexuronate/mmol creatinine); (mean ± 95% CI)] in the early morning urine (EMU) of stone-formers (SF), post-treated SF, and normal controls

Normal individuals excreted higher amount of urinary GAGs (233.3 ± 80.8) than stone-formers (SF) (144.7 ± 65.86) although the difference is not statistically significant (Figure 4.2). A significant reduction in the urinary GAGs in post-treated SF (109.7 ± 44.28) was found when compared with normal subjects.

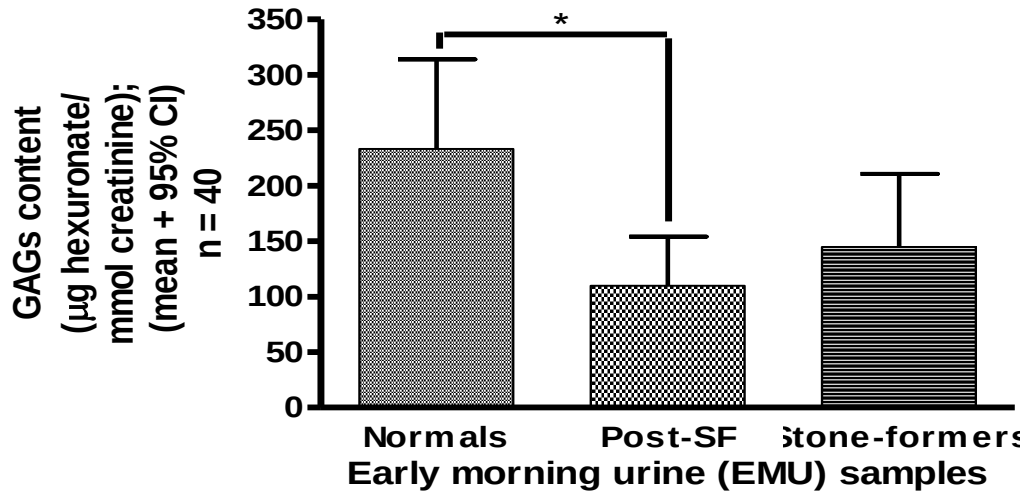

Figure 4.2. The total glycosaminoglycans (GAGs) contents standardized against creatinine (mean + 95%CI) from early morning urine samples of normal subjects, stone-formers (SF) and post-treated SF.

4.4.2 Urinary hyaluronan (HA) excretion [(ng/mmol creatinine); (mean ± 95% CI)]

SF had an enhanced urinary excretion of HA (1721 ± 1191) but no increase of urinary HA in post-treated SF group (681.1 ± 258.8) was found when compared with the normal individuals (811 ± 401.5) (Figure 4.3).

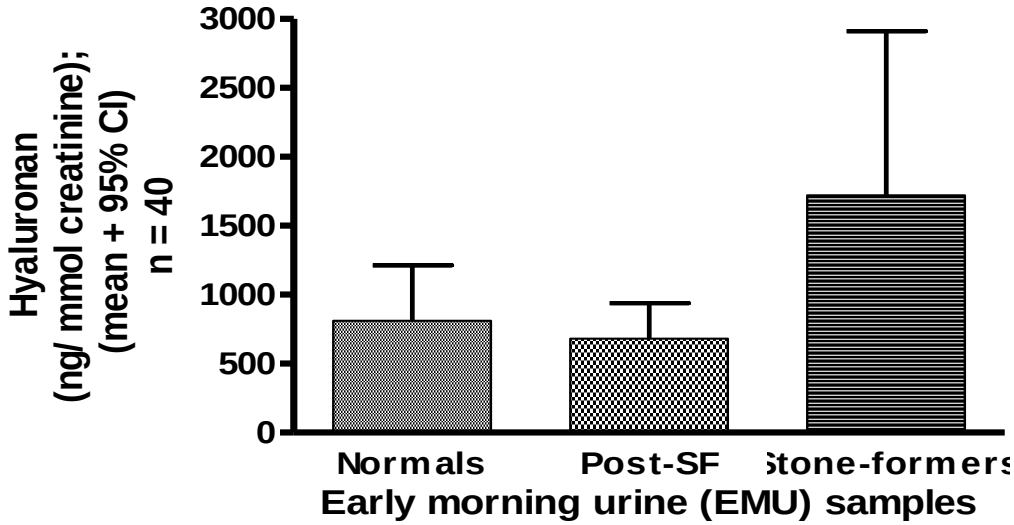

Figure 4.3. The hyaluronan (HA) concentrations standardized against creatinine (mean + 95%CI) from early morning urine samples of normal subjects, stone-formers (SF) and post-treated SF.

4.4.3 Proportion of HA in total GAGs [%; (mean ± 95% CI)]

SF (0.77 ± 0.37) had a higher HA proportion of total GAGs (Figure 4.4) while post-treated SF (1.09 ± 0.76) had a significant higher proportion of HA of total GAGs than normals (0.28 ± 0.08).

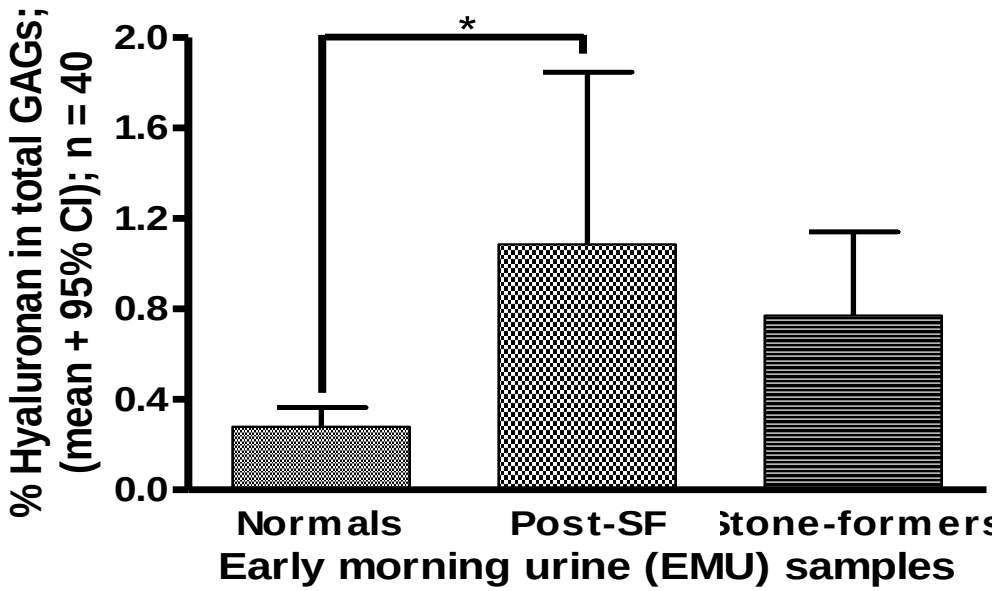

Figure 4.4. The percent of hyaluronan (HA) in total glycosaminoglycans (GAGs) (mean + 95%CI) from early morning urine samples of normal subjects, stone-formers (SF) and post-treated SF.

4.5 Discussion

Increased supersaturation of urine, enhancement of urinary crystallization promoters and reduction of crystallization inhibitors lead to nucleation, growth and aggregation of calcium oxalate (CaOx) crystals [1] that results in renal stone formation. Glycosaminoglycans (GAGs) are one of the major macromolecular modifiers on CaOx crystallization.

GAGs in the urine can be derived from shedding from the surface of the tubular epithelial lining of urinary tract, excreted from the glomerular basement membrane [17], and from glomerular filtration. Results of this study correlated with previous studies [8-10] that normal individuals excreted higher amount of urinary GAGs than stoner-formers (SF) although the difference is not statistically significant (Figure 4.2). It also found that there is a significant reduction in the urinary GAGs in stone-former after extracorporeal shock wave lithotripsy (ESWL) (post-treated SF) (Figure 4.2). This reduction of GAGs was real as the GAGs were standardized with creatinine to eliminate the dilution effect of increased intake of water which is usually recommended by urologists after treatment. The possible explanation of the reduction of GAGs after ESWL may be due to the treatment induced epithelial damage on stone binding site of urinary lining during stone removal that results in the release of enzymes that cleave GAGs and also the expression of GAGs binding proteins on injured cell surface such as CD44 for HA binding. Thus, free and high molecular weight GAGs in urine that available for extraction and detection reduced.

GAGs are highly negatively charged polysaccharides with repeated disaccharides units and presence of carboxyl or sulphate groups lead to the stretched or extended conformation of polysaccharide chains of GAGs [18] as an expanded random coil structure [19]. Addition of GAGs can significantly inhibit the adherence of CaOx monohydrate (COM) crystals to renal epithelial cells and this was shown from studies of Madin-Darby canine kidney (MDCK) [20, 21] and nontransformed African green monkey cells (BSC-1) [22] by coating onto the crystals instead of coating onto the cells [22]. Semi-synthetic polysaccharides (SSPs) that mimic the structure of GAGs are potent inhibitors for the crystal-cell interaction on the COM binding on the MDCK cells [20] when the crystals were pretreated with SSPs but not the pretreated cells. The increased production of GAGs by tubular epithelial cells may protect cells from toxic effects of calcium oxalate crystals and oxalate ions [7]. Therefore, GAGs can prevent the binding of CaOx crystals onto the negatively charged urinary epithelial cells surface due to the charge repulsion and the steric hindrance by the GAGs covering the surface of the crystals and thus preventing the crystals to be retained and becoming a potential stone. GAGs can also enhance the urinary supersaturation by forming complex with calcium salts [23] so that higher concentration of calcium can be tolerated in the urine without precipitation of CaOx crystals. The higher GAG content in normal population can be protective against renal stone disease by (1) preventing CaOx crystals binding to the renal epithelial cells surface as well as (2) reducing the CaOx crystals being formed through raised calcium holding in urine. Reduced excretion of GAGs in SF and the lower GAGs amount in the post-treated SF group is a risk factor for the occurrence and recurrence of stones.

Hyaluronan (HA) is found in urine [13, 24] and the stone matrix [25, 26]. It is a non-sulfated GAG involved in several fundamental cell biological processes such as regulation of cell-cell adhesion, development, proliferation, migration, differentiation, metastasis, inflammation, and wound healing [27]. HA fragments are released into urinary tract [28] as a consequence of active turnover of renal tissue in the diseased state. Studies showed that migrating cells produce large amounts of HA during repair of damaged renal epithelial cells [29, 30]. Up-regulation of HA was observed in human kidney proximal epithelial (HK-2) cells during CaOx crystals induced cell injury [15] for mediating repair of an injured epithelium.

Active SF had an enhanced urinary excretion of HA but no increase of urinary HA was found in post-treated SF group (Figure 4.3). This can be explained that active SF had CaOx induced cell injury occurring and this enhanced the excretion of HA (through inflammatory response) while in post-treated SF, there was no cell injury to trigger the excretion of HA as the stone was removed by ESWL and the wound caused by stone and ESWL had healed already 2 weeks after treatment. A long-term follow-up of the post-treated SF may provide more information on the recurrence of renal stone disease due to the presence of residual subclinical inflammation and/or clinically insignificant residual fragments (CIRF) after ESWL treatment.

However, when the proportion of HA in total GAGs is investigated, it was found that active SF had higher proportion of HA in total GAGs (Figure 4.4) that correlated with previous study [13]. It was also interesting to note that post-treated

SF had a significant higher proportion of HA in total GAGs (Figure 4.4) than normals although the HA concentrations in post-treated SF was no different from normal individuals. This higher proportion of HA from total GAGs in post-treated SF is suggested to be due to the fact that they had the lower total GAGs amount.

HA produced is known to be bound to its receptors CD-44 on the surface of renal epithelial cells [31]. This causes the cell surface to become sticky and enhance the crystal-cell binding in cell culture model [22, 32]. HA has been found to bind CaOx crystals at the surface of proliferating renal tubular cells in cell culture model. It acts as a crystal-binding molecule at the surface of MDCK [33] and BSC-1 [22] cells which are involved in the adherence of COM crystals to the renal tubular epithelium, and considered as a critical event in the pathophysiology of calcium nephrolithiasis. Previous study found that HA secreted by the injured HK-2 cells lead to the adherence of CaOx crystals to the injured epithelial cells and results in the internalization of crystals [15]. This suggested that increased HA production during inflammation of renal epithelial cells in SF does enhance the risk of renal stone formation and higher HA proportion in total GAGs of both active SF and post-treated SF indicated that they have a higher risk for the occurrence and recurrence of renal stones due to the crystallization-promoting property of HA. It is suggested that HA becomes an accidental participant in the pathogenesis of stone disease and patients who have undergone procedures or treatments, may have compromised the integrity of the urothelium lining with HA being subsequently being released as an inflammatory response molecule. This can be a useful and a potential diagnostic marker.

4.6 Conclusion

Post-treated SF and active SF groups had lower total GAGs content but higher proportion of HA than that of the normal controls indicating that urinary GAGs and HA are probably a protective and risk factors respectively for the renal stone disease. The higher occurrence and recurrence rate of renal stone disease in active SF and post-treated SF may be due to the lower GAG amount but higher proportion of HA in their urine, indicating an underlying inflammatory response which leads it to become an accidental participant in the pathogenesis of renal stone.

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Chapter 5

Enhanced hyaluronan secretion and CD44 expression of human kidney proximal epithelial (HK-2) cells under inflammatory condition and calcium oxalate induced cell injury

5.1 Abstract

Background: CD44 is a widely expressed cell adhesion molecule that can interact with hyaluronan (HA) at inflammatory sites for wound healing. Therefore, interaction between CD44 and HA can be demonstrated to be involved in sub-clinical renal stone diseases. CD44 expression and HA secretion were studied in human kidney proximal epithelial (HK-2) cells by stimulation of an inflammatory cytokine – interleukin-1 beta (IL-1β) that mimics the inflammatory response during cell injury. HA secretion by calcium oxalate (CaOx) induced cell injury of HK-2 cells were also investigated in a two-compartment culture system.

Methods: HK-2 cells were grown to confluence and stimulated under serum-free medium condition with IL-1β for mimicking inflammatory cell injury. CD44 expression was examined by flow cytometry and HA secretion was studied by enzyme-linked immunosorbent assay (ELISA). Physiological relevant concentrations of calcium (12 mmol/L) and oxalate (2.2 mmol/L) in renal tubules were added into the apical layer of HK-2 cell monolayer to induce CaOx crystallization and the HA secretion from the

HK-2 cells due to the CaOx crystals induced injury was measured by high performance liquid chromatography (HPLC).

Results: Stimulation of HK-2 cell with IL-1β led to a significant and dose-dependent up-regulation of HA excretion as well as CD44 expression in the culture medium and HK-2 cells, respectively. HA excretion was observed in both the apical and basal culture medium after CaOx induced HK-2 cell injury.

Conclusion: This study suggested the possible events during sub-clinical CaOx renal stone disease. HA was secreted during the CaOx induced cell injury and the injured cells also secreted IL-1β as the inflammatory cytokine that can eventually lead to further HA secretion as well as CD44 expression that can interact with the HA being secreted for CaOx binding to the injured and/or regenerating renal cells.

5.2 Introduction

The retention of crystals within renal tubules provides enough time for a crystal to grow continuously to a full-sized stone. Retention can occur due to crystals adhering to injured and repairing tubular cell surfaces [1]. Thus, cell injury for crystal adhesion [2, 3] is considered as the important first step in the formation of calcium oxalate renal stones.

In this study, the human kidney proximal epithelial (HK-2) cells was stimulated by an inflammatory cytokine – interleukin-1 beta (IL-1β) to mimic the inflammatory response during cell injury and observed the cellular response by measuring the hyaluronan (HA) secretion and CD44 expression of cells.

HA is involved in regulation of cell-cell adhesion, inflammation, and wound healing [4] and studies have shown that migrating cells produce large amounts of HA during repair of damaged renal epithelial cells [5, 6]. Thus, HA may be secreted from the stimulated HK-2 cells under inflammatory response to mediate the repairing of an injured epithelium. This may affect the CaOx crystallization as HA was shown to have crystallization-promoting properties [7, 8].

HA produced, is known to bind to its receptors CD44 on the surface of renal epithelial cells [9]. This causes the cell surface to become sticky and enhance the crystal-cell binding in cell culture model [10, 11]. Thus, CD44 expression of HK-2

cells (under inflammatory response) may be up-regulated and favour the crystal binding and retention on cell surface for stone formation by CD44-HA interaction.

A two-compartment culture system was established (Figure 5.1) with intact HK-2 cell monolayer that displays similarly the apical and basal sides of the urothelial lining by providing urinary environment (apical) for calcium oxalate induced cell injury study and nutrient (basal) for maintaining cell growth simultaneously. When physiological relevant concentrations of calcium and oxalate are added to the apical side of HK-2 cell monolayer, CaOx crystals are expected to form and bind to the injured HK-2 cell monolayer. This may eventually lead to the secretion of HA as a mediator for repairing of an injured epithelium. Therefore, an increase in HA secretion can interact with CD44 expressed on the injured and/or regenerating renal cells and facilitate further CaOx binding and thus pose a risk for renal stone formation.

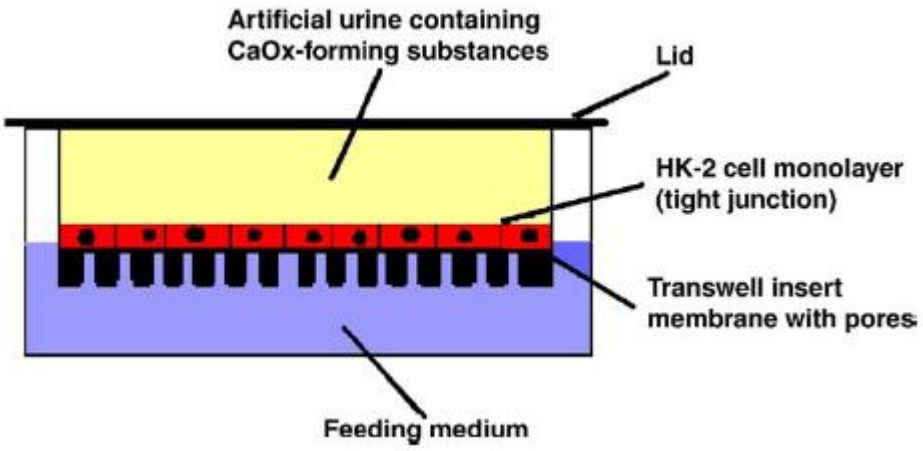

Figure 5.1. The two compartment culture system with intact HK-2 cell monolayer cultured on the transwell insert that displays similarly the apical and basal sides of the urothelial lining by providing urinary environment (apical) for calcium oxalate induced cell injury study and nutrient (basal) for maintaining cell growth simultaneously. (Yuen JWM *et al* 2010)

5.3 Materials and methods

5.3.1 Interleukin-1 beta (IL-1β) to mimic inflammatory response on to human kidney proximal epithelial (HK-2) cells

HK-2 cell line (CRL-2190; ATCC, VA, USA) is the proximal tubular cell (PTC) line derived from male normal human kidney with transduction of human papilloma virus 16 (HPV-16) E6/E7 genes and cultured in complete medium [F12-Ham enhanced Dulbecco's modified eagle's medium (DMEM) (Sigma, MO, USA) with 10% fetal bovine serum (FBS) (Gibco, NY, USA)] at 37℃ with saturated humidity and 5% carbon dioxide. Cultures were grown until confluent and re-seeded with 10^5 cells into 25 cm^2 BD Falcon cell culture flask with 0.2 μm filter cap (BD Biosciences, NJ, USA) for experiments.

Re-seeded HK-2 cells were serum deprived for 48 hours. Growth arrested cells were subsequently stimulated with recombinant IL-1β (Perpotech, NJ, USA) at concentrations 0, 0.5, 1.0, 5.0, 10.0 ng/mL under serum-free conditions for 24 hours before experiments.

5.3.2 HA measurement by HA-ELISA

The Hyaluronan Enzyme-Linked Immunosorbent Assay Kit (HA-ELISA; echelon biosciences, UT, USA) is a quantitative enzyme-linked immunoassay designed for the

in vitro measurement of HA levels in cell-culture supernatants. It is a competitive ELISA assay in which the colorimetric signal is inversely proportional to the amount of HA present in the sample.

Culture media from IL-1β stimulated HK-2 cell cultures were collected and centrifuged at 1000 rpm for 5 minutes at 4℃. Culture supernatants and HA standards were first mixed with the Detector, then added to the HA ELISA Plate for competitive binding. An enzyme-linked antibody and colorimetric detection at 405 nm was used to detect the HA detector bound to the plate. A standard curve of percent binding versus concentrations of HA standards was plotted using linear regression. HA concentration in culture supernatants was determined by comparing their percentage of binding relative to the standard curve. All samples and standards were performed in triplicate.

5.3.3 CD44 immuno-phenotyping by flow cytometry

IL-1β stimulated HK-2 cell cultures were harvested with 500 μL of 0.02% trypsin with 2 mM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS) and resuspended in 4 ml PBS with 2% FBS. They were then centrifuged at 1000 rpm for 5 minutes at 4℃ and discarded the supernatants. Cells were then resuspended with 50 μL PBS with 2% FBS and incubated with 12 μg/mL fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-CD44 antibody (Immunotech, USA) for 30 minutes at 5℃ in dark. FITC-labelled cells were resuspended in 2ml PBS with 2% FBS

and analyzed by flow cytometry with 495 nm excitation and 520 nm emission wavelengths.

5.3.4 Establishment of intact HK-2 monolayer cells with tight junctions

Logarithmically growing HK-2 cells were plated at density of 5 x 10^3 cells/insert on 0.4 μm and 11.2 cm^2 permeable polycarbonate transwell microporous filter membranes (Coster Corning, NY, USA) and allow to grow for 7 to 10 days until confluence with complete medium on both insert (apical) and feeding (basal) sides of transwell. Intact monolayer was subjected for AC impedance analysis by using Ametek Versa STAT3 electrochemical impedance spectroscopy with Ametek V3-studio v1.0.2711 (Princeton applied research, TH, USA) to confirm the tight junctions of HK-2 cells. Besides, 1 mg/mL 10 kDa FITC-dextran (Sigma, MO, USA) in DMEM/F12 with penol red (Sigma, MO, USA) was added into the apical side of transwell the cells for 3 hours in order to confirm the tight junctions of cells by the absence of trans-epithelial flux. This can be done by measuring the FITC fluorescence signals of media collected from both apical and basal compartments by Tecan SpectraFluor Plus microplate reader (Tecan, Grodig, Austria) with 485 nm excitation and 535 nm emission wavelengths.

5.3.5 Calcium and oxalate solutions preparation

Calcium and oxalate solutions were prepared by dissolving calcium chloride (BDH, Poole, England) and sodium oxalate (Sigma, MO, USA) in artificial urine (AU) accordingly. The constituents of the AU used in this study were as described in Kavanagh's study [12]. All chemicals were of analytical grade. The chemicals of AU were dissolved in MilliQ water and then the pH was adjusted to 6.0 with hydrochloric acid.

5.3.6 Calcium oxalate induced HK-2 cell injury

Physiological relevant concentrations of calcium (12 mmol/L) and oxalate (2.2 mmol/L) along renal tubules were added to the apical side of intact HK-2 cell monolayer to mimic the hypercalciuria and hyperoxaluria conditions for CaOx crystallization. Briefly, 250 μL of 12 mmol/L calcium and equal amount of 2.2 mmol/L oxalate in AU were added separately into the apical side HK-2 cells (insert well) to initiate CaOx crystallization and immediately followed by orbital shaking at 37℃ for 10 minutes. The incubation solution was then removed from the transwell and washed thrice with PBS followed by replacement of complete media for further 18 hours post-CaOx induced cell injury incubation to allow HA secretion for cell repairing.

5.3.7 Recovery of glycosaminoglycans (GAGs) in culture media

GAGs were recovered by a protocol previously established [7]. Briefly, culture medium was centrifuged at 1500 rpm for 15 minutes to remove the cell debris. Supernatant collected was diluted with 3 volumes of 0.025 M sodium acetate buffer at pH 5.8. Then, 0.1 volume of 5% cetylpyridinium chloride (CPC) in acetate buffer was added into the buffered supernatants. The precipitate was washed with cool distilled water and then dissolved in propanol. 4 volumes of sodium acetate-saturated ethanol (EtOH) were added to precipitate the urinary polyanionic macromolecules as sodium salts. GAGs were then recovered by the papain (Sigma Chemical Co., St. Louis, USA) digestion of the precipitate at 65℃ followed by another sequential CPC-EtOH precipitation.

5.3.8 Double enzyme digestions of GAGs for HA disaccharides

Individual GAGs extracts from culture supernatants and HA from Pig skin (Seikagaku Corporation, Tokyo, Japan) for HA standards were digested sequentially with 0.3 turbidity reducing units of hyaluronidase from *Streptomyces hyalurolyticus* (Seikagaku Corporation, Tokyo, Japan) and 0.1 U of chondroitinase ABC from *Proteus Vulgaris* (Seikagaku Corporation, Tokyo, Japan) as previously described [7] to yield the HA disaccharides.

5.3.9 HA disaccharides measurement by high performance liquid chromatography (HPLC)

HA disaccharides quantitation was performed by a Waters HPLC system (Waters Corporation, Milford, USA) comprising a Waters Alliance separation module 2695, a Waters photodiode array detector 2996, and an Empower Pro software (Waters Corporation, Milford, USA). The settings on the HPLC system was based on the previous protocols [7] with modifications. Double enzyme digested samples and HA standards were reconstituted with mobile phase and injected into a 250 x 4.6 mm Econosphere NH_2 5µm column (Grace, Deerfield, USA) connected with a 7.5 x 4.6 mm Econosphere NH_2 5µm Alltech guard column (Grace, Deerfield, USA) with 5mM sodium dihydrogen orthophosphate/orthrophosphoric acid (pH 2.60) as mobile phase at a flow rate of 1 mL/minute. The column eluate was monitored at 232 nm and the peak area were determined. The retention time for HA disaccharides of processed samples and HA standards were determined at 7.5 minutes. HA disaccharides in samples were detected from HA standard curve.

5.3.10 Statistical analysis

GraphPad Prism version 4.03 for window (GraphPad Software, San Diego California, USA) was used to perform all statistical analysis in this experiment. One-way analysis of variance (ANOVA) was used for the comparison of the mean differences of hyaluronan and CD44 after HK-2 cells were stimulated by different concentrations of IL-1β. A *P* value of less than 0.05 was considered to be statistically significant. All significant ANOVA test results were analysis by Dunnett's multiple comparisons post-test (IL-1β stimulated groups versus control group). The P value of less than 0.05 and 0.01 were specified by the symbols * and ** respectively in the graphs.

5.4 Results

5.4.1 The hyaluronan (HA) standard curve of the HA-ELISA

HA standard concentrations (Figure 5.2) were inversely proportional ($r^2 = 0.9321$) to the % binding of competitive HA detector as analyzed by linear regression.

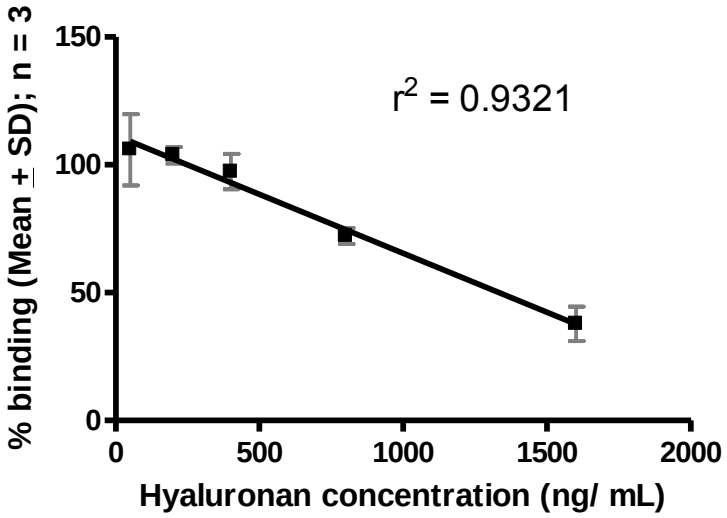

Figure 5.2. The hyaluronan (HA) standard curve. HA concentrations were inversely proportional to the % binding of competitive HA detector.

5.4.2 Hyaluronan (HA) secretion by IL-1β stimulated HK-2 cells

The addition of inflammatory cytokine – IL-1β to HK-2 cells mimics the inflammatory response resulting in a dose dependent HA secretion from HK-2 cells (Figure 5.3).

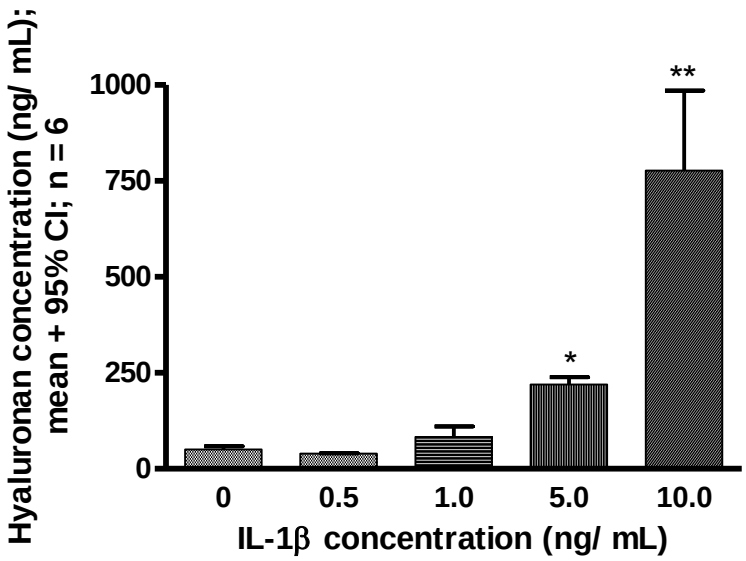

Figure 5.3. Dose-dependent increased in hyaluronan (HA) secretion by IL-1β stimulated HK-2 cells.

5.4.3 CD44 expression by IL-1β stimulated HK-2 cells

HK-2 cells is stimulated by IL-1β as shown in a dose dependent enhancement of CD44 expression on HK-2 cell surface (Figure 5.4).

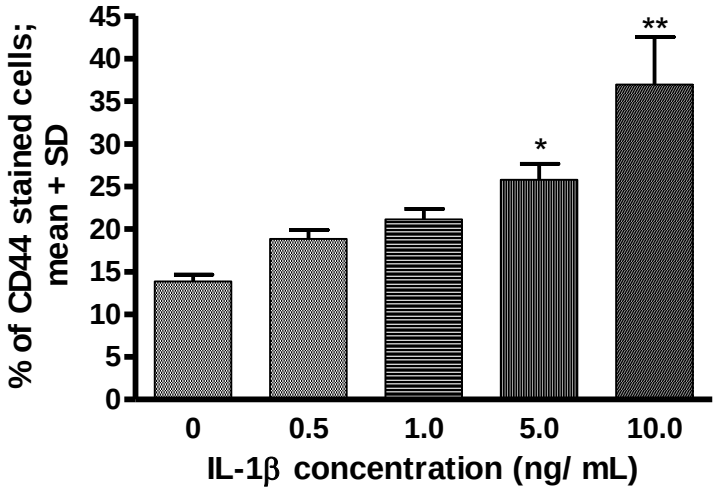

Figure 5.4. Dose-dependent increased in CD44 expression by IL-1β stimulated HK-2 cells.

5.4.4 The hyaluronan (HA) standard curve as determined by HPLC

HA standard concentrations (Figure 5.5) were directly proportional ($r^2 = 0.9999$) to the peak area of the distinct HA peak observed at 7.5 minutes with maximum absorbance at 232 nm as analyzed by linear regression.

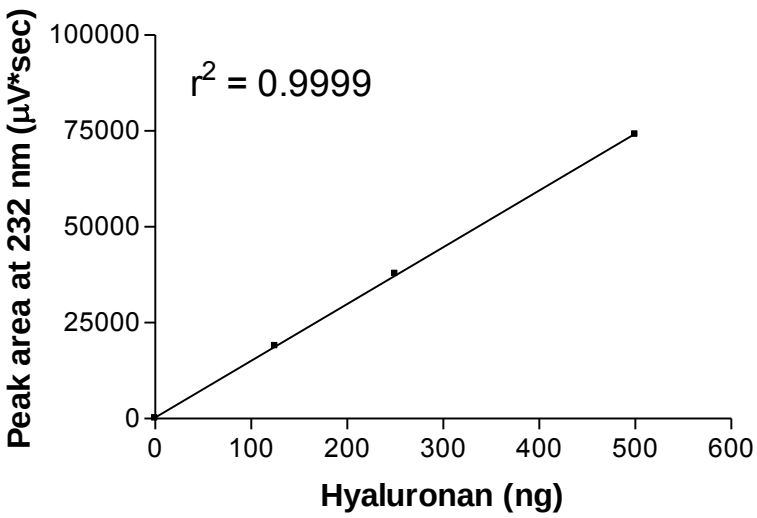

Figure 5.5. The hyaluronan standard curve as determined by HPLC. Peak area of the distinct peak observed at 7.5 minutes with maximum absorbance at 232 nm were measured.

5.4.5 HA secretion by HK-2 cells under CaOx induced cell injury

Similar concentrations of HA disaccharides were detected (Figure 5.6) in the harvested media collected from the apical (214.4 ng/mL) and basal (256.3 ng/mL) sides of HK-2 cell monolayer after injury by CaOx crystals.

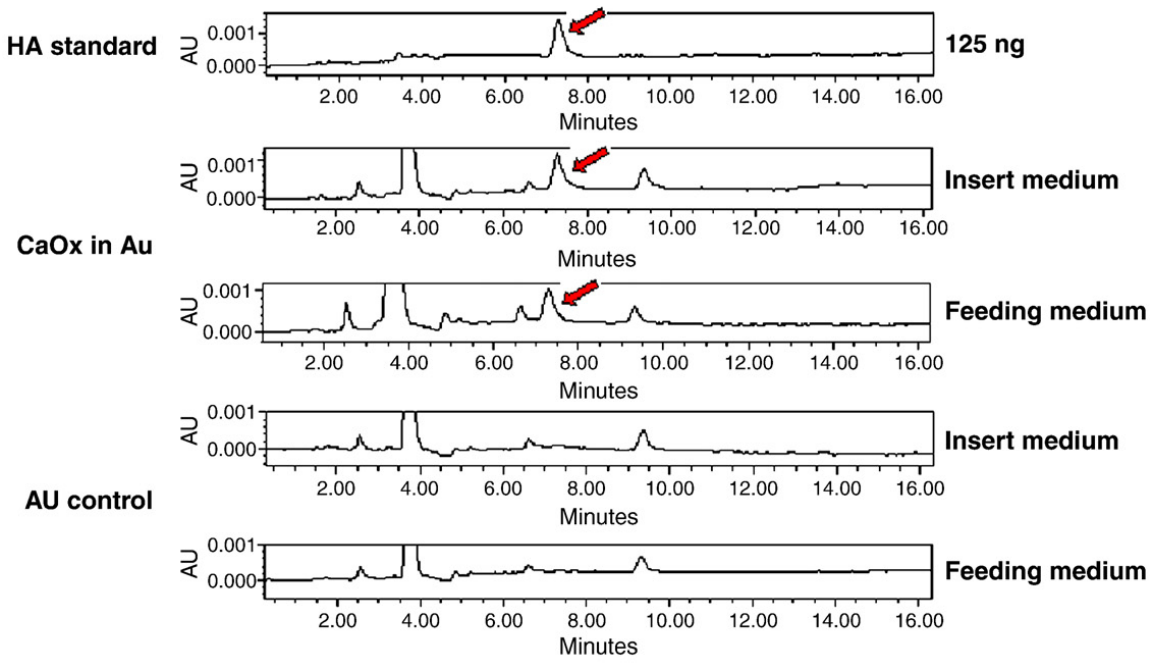

Figure 5.6. Chromatograms of hyaluronan (HA) disaccharides after sequential *Streptomyces* hyaluronase and chondroitinase ABC digestions of 125 ng HA standard, culture supernatants of feeding (basal) and insert (apical) sides of enzyme-digested calcium oxalate (CaOx) induced HK-2 culture, and controls without the addition of CaOx [artificial urine (AU) only] in HK-2 culture. Retention time for HA disaccharides were determined at 7.5 minutes from the HA standard.

5.5 Discussion

The retention of crystals within renal tubules is the main cause for the development of renal stones because only a retained crystal can have enough time to grow continuously and become a full-size stone. Retention can occur due to crystals adhering to tubular cell surfaces. An intact and functional epithelium formed by renal tubular cells with characteristics of the late nephron is non-adherent to crystals [1, 13]. However, the non-adherent property of the renal tubular cells is lost after epithelial injury and during repair [1]. Thus, cell injury is a prerequisite for crystal adhesion [2, 3] and the formation and subsequent retention of large particles in renal tubules is to be expected after cell injury and considered as the important first step in the formation of calcium oxalate renal stones.

Interleukin-1 beta (IL-1β) is the first cytokine being secreted during cell injury to mediate the immune response. Therefore, the human kidney proximal epithelial (HK-2) cells was stimulated by IL-1β to mimic the inflammatory response during cell injury and observed the cellular response by measuring the hyaluronan (HA) secretion and CD44 expression of cells.

HA is found in urine [7, 14] and the stone matrix [15, 16] and it is a non-sulfated glycosaminoglycans (GAG) involved in several fundamental cell biological processes such as regulation of cell-cell adhesion, development, proliferation, migration, differentiation, metastasis, inflammation, and wound healing [4]. Studies showed that

migrating cells produce large amounts of HA during repair of damaged renal epithelial cells [5, 6]. HA was shown to have crystallization-promoting properties [7, 8] and dose-dependent up-regulation of HA by human kidney proximal epithelial (HK-2) cells stimulated with IL-1β (Figure 5.3) was observed. Thus, HA was secreted form the stimulated HK-2 cells under inflammatory response that acts as a mediator for repairing of an injured epithelium.

HA produced, is known to be bind to its receptors CD44 on the surface of renal epithelial cells [9]. This causes the cell surface to become sticky and enhance the crystal-cell binding in cell culture model [10, 11]. Dose-dependent enhancement of CD44 expression of HK-2 cells under IL-1β stimulation (Figure 5.4) was also found in this study indicated that HK-2 cells under inflammatory response favor the crystal binding and retention on cell surface for stone formation by CD44-HA interaction. HA has been found to bind CaOx crystals at the surface of proliferating renal tubular cells in cell culture model. It acts as a crystal-binding molecule at the surface of MDCK [17] and BSC-1 [10] cells which is involved in the adherence of COM crystals to the renal tubule epithelium, and considered as a critical event in the pathophysiology of calcium nephrolithiasis.

A two-compartment culture system was established with intact HK-2 cell monolayer that mimics the apical and basal sides of the urothelial lining. When physiological relevant concentrations of calcium and oxalate were added to the apical side of HK-2 cell monolayer, CaOx crystals were formed and bound on the apical

surface of injured HK-2 cells immediately after 10 minutes shaking [18]. Other studies showed that membrane fragments of renal epithelial cells promote crystallization from supersaturated calcium oxalate solutions [19] and the adherence of calcium oxalate crystals increases with the number of crystals and shows concentration-dependent saturation [20].

It is important that HK-2 cells triggered the secretion of HA (Figure 5.6) in response to CaOx induced cell injury as a mediator for repairing of an injured epithelium. Thus, an increase in HA secretion can interact with CD44 expressed on the injured and/or regenerating renal cells and facilitate further CaOx binding and thus pose a risk for renal stone formation.

5.6 Conclusion

This study suggested the possible events during subclinical CaOx renal stone disease. HA was secreted during the CaOx induced cell injury and the injured cells will also secrete IL-1β as the inflammatory cytokine that can eventually lead to further HA secretion as well as CD44 expression that can interact with the HA being secreted for CaOx crystal attachment and retention for stone formation during the healing of the injured kidney cells.

5.7 References

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Chapter 6

Melamine crystallization: the physiochemical properties, interactions with others lithogenic salts and response to therapeutic agents

6.1 Abstract

Background: There were reports of children in China being admitted with renal stones and/or renal failure by 2007 that were caused by melamine and its co-contaminant cyanurate. This study aimed to investigate the physicochemical behaviour of melamine as a foreign entity in urine, its interaction with other endogenous urine factors and response to the therapeutic agents in the renal environment in-vitro.

Methods: A Mixed Suspension Mixed Product Removal system was the in-vitro set-up for crystallization studies of melamine in artificial urine. Crystallization kinetic parameters including nucleation rate, growth rate and suspension density were determined according to the crystal number and size measured by a particle counter.

Results: Melamine crystallized out from artificial urine under normal urinary pH conditions (pH 5.0 – 6.5) but crystallization was strongly inhibited at pH 4.5 or lower. Presence of melamine significantly enhanced the precipitation of calcium oxalate while the presence of uric acid significantly reduced melamine crystallization. The presence of bacteria mimicking urinary tract infection promoted the melamine crystallization.

Clinical relevant drugs such as citrate and bicarbonate did significantly reduce melamine crystallization. Traditional Chinese medicines such as Shi Wei can acutely inhibit melamine crystallization but its inhibitory effect was reduced after prolong treatment.

Conclusions: Melamine crystallizes in acidic urine and can interact with other lithogenic salts. Urinary tract infection promotes melamine crystallization. Citrate and bicarbonate therapy were effective against melamine crystallization. Shi Wei can also act as a supportive treatment for quick and acute relief of melamine stone formation.

6.2 Introduction

Since 2004, acute renal failure in animals was found to be associated with adulterated pet food in Asia and United States [1]. Melamine was found contaminated in the wheat gluten added to pet food. A co-contaminant, cyanuric acid was also identified [2]. In early September of 2008, there were confirmed reports of renal stones in infants and children, admitted in hospitals in China. Subsequently, melamine was found added into milk, in an attempt to increase nitrogen content (66.6% by weight) during classic crude protein test (Kjeldahl and Dumas method) for dairy products so as to increase the measured protein content. At the time, 6 infants were reportedly to have died and around 300,000 suffered from urinary tract ailments including renal stones, 850 are still being treated and 150 are seriously ill [3]. In Hong Kong, 15 children had tested positive and over 40,000 had been screened to be healthy [4,5]. A study of 3,835 children attending Princess Margaret Hospital in Hong Kong were further investigated and 22 (0.6%) showed renal disorders but not necessarily related to melamine [6]. Most children afflicted with melamine-related stones were described as asymptomatic [7] until renal abnormalities were severe enough to cause impaired renal function by which time melamine and its crystalline stone had done its damage. Hence, the physicochemical nature of melamine in body fluids (blood and urine) and its handling is largely unknown until recently. Recent reports suggest that the levels of melamine and cyanurate allowed by the World Health Organization (WHO) should be lowered for infants (< 3-years old), as the relative risk for renal stones was 1.7 compared to control at the previously defined safe levels (< 0.2 mg/kg per day) [8].

Many in-vitro methods are used to study urine crystallization. The mixed suspension, mixed product removal (MSMPR) adopted here and already established, reaches a steady state supersaturation and a good model for in-vitro renal crystallization studies [12]. This model is useful to investigate how melamine and cyanuric acid behave by changing urinary factors such as supersaturation and pH, as well as in the presence of other stone forming salts including calcium oxalate (CaOx), calcium phosphate (CaP) and uric acid (UA) because they are important factors that may affect melamine crystallization [13-15]. This information would be vital as melamine is still allowable in foods and meats for human consumption according to the United States Food and Drug Administration (FDA). Furthermore, wide use of melamine in fertilizers is a risk factor and we will determine whether such allowable limits pose a crystallization risk index which would potentially form renal stones.

Urinary tract infection (UTI) is one of the most common bacterial infections in children [16]. 8% of girls and 2% of boys suffered from UTI in childhood [17]. Since melamine stones occurred in infants and child, UTI may contribute to melamine crystallization. *Escherichia coli* (*E. coli*) is the main bacterium (about 75% of UTI) isolate from urine [18]. Thus, the effect of *E. coli* on melamine crystallization was tested.

Potassium citrate and sodium bicarbonate are used clinically in the treatment of kidney stones. Urinary citrate forms a soluble complex with calcium by chelation that inhibits the formation and propagation of calcium-containing crystals [19] while

bicarbonate enhances the urinary pH [20] to treat uric acid stones (alkaline treatment). These two therapeutic agents were also administered to children with melamine stones. Therefore, the investigation of citrate and bicarbonate on the melamine crystallization can also suggest the usefulness of current therapy protocol to children.

Traditional Chinese medicines (TCM) have been used for treating renal stones due to their anti-lithogenic activities. Previous study found that a Chinese herb Shi Wei (Folium Pyrrosiae) has a potential to inhibit urinary crystallization by reducing the urinary specific gravity and enhancing the urinary magnesium. Reducing the specific gravity of urine implied the reduction of urinary supersaturation and enhancing the urinary magnesium, which is an inhibitor of crystallization, that can complete with calcium to form ion complexes with oxalate (MgOx) which is more soluble than CaOx. Thus, this study aims to understand the effects of Shi Wei on melamine crystallization to see whether Shi Wei may also be a suitable therapeutic agent for the prevention of the occurrence and recurrence of melamine stones in infants.

6.3 Materials and methods

6.3.1 Artificial urine (AU) and reagents preparation

The constituents of AU used in this study (Table 3.1) were as described in Kavanagh's study [21]. AU was freshly prepared daily and all chemicals were of analytical grade. The chemicals of AU were dissolved in MilliQ water and then the pH was adjusted to 6.0 with hydrochloric acid. 10 mM stock melamine solution was prepared by dissolving 1.2612 g of melamine (Sigma-Aldrich, US) in 1 L of MilliQ water. 10 mM stock cyanuric acid solution was prepared by dissolving 1.2908 g of cyanuric acid (Sigma-Aldrich, China) in 1 L of MilliQ water.

6.3.2 Mixed suspension, mixed product removal (MSMPR) system set up for melamine crystallization

Two reduced size (20 mL) crystallizers [12] in parallel (test and control chambers) had been developed for crystallization studies with AU. The crystallizer is a dual-layer glass beaker for which water can pass through the in-between layer so that it can be kept at 37℃ throughout the experiments by the water circulation through a 37℃ water bath and water-jacketed pumping system. A close fitting lid covered the opening of the crystallizer to minimize evaporation and with 4 openings for the fitting of inlet of AU, melamine solution, cyanuric acid solution, and outlet tubes for mixed product removal. The suspension inside the crystallizer was mixed by a magnetic stirrer with the magnetic plate under the crystallizer. A flow rate of 2.64 mL/minute for the AU and

0.11 mL/minute for the melamine and cyanuric acid solutions were applied so that the flow rate at the outlets of the crystallizer was the sum of the three feed solutions that is 2.86 mL/minute with the proportion of 92% AU and two 4% feed solutions of melamine and cyanuric acid. The whole set up of MSMPR system was shown on Figure 6.1.

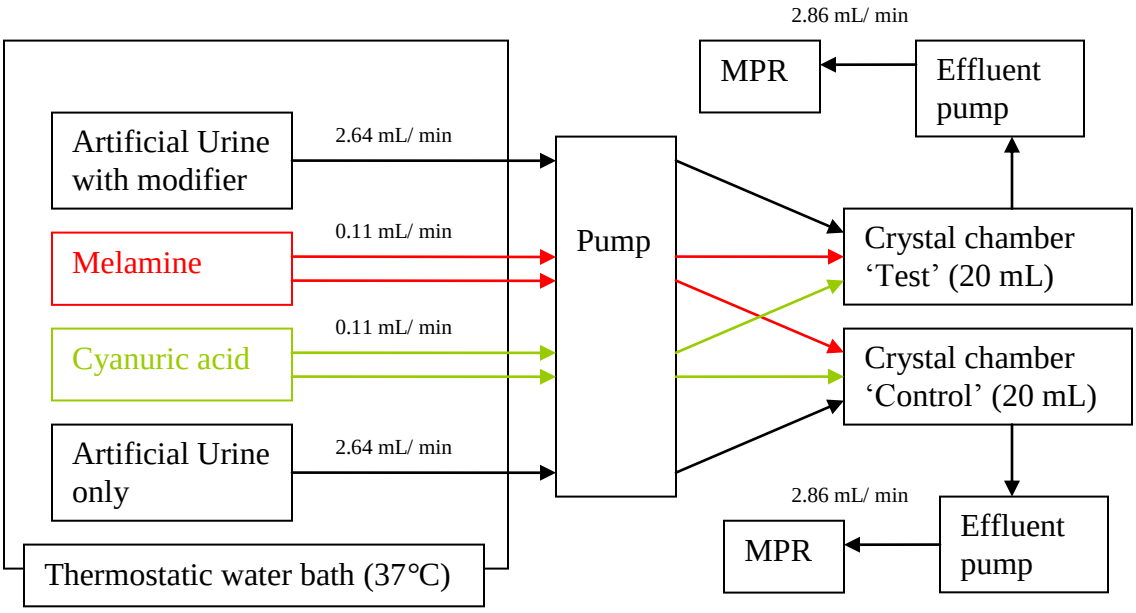


Figure 6.1. Schematic diagram of the MSMPR crystallizer for melamine crystallization. Artificial urine (AU), melamine and cyanuric acid were added into both test and control chambers from three inlet tubes. Modifiers such as other lithogenic salts and therapeutic agents for testing were added into the test AU. An outlet tube from each chamber was for mixed product removal for crystal size and number measurement by a particle counter. A flow rate of 2.64 mL/minute for the AU and 0.11 mL/minute for the melamine and cyanuric acid solutions were applied so that the flow rate at the outlets of the crystallizer was the sum of the three feed solutions that is 2.86 mL/minute to keep the equilibrium volume of urine inside the chamber throughout the run.

The three feed solutions were equilibrated to 37℃ before the experiments were begun. The two chambers were then run simultaneously for 7 to 8 residence times [21,22] for equilibration of the MSMPR system. The crystal number and size were found to remain constant after 8 residence times. After that, 10 consecutive measurements of crystal numbers and sizes were obtained with 7 minutes intervals for each chamber by using the Coulter Multisizer 3 (Beckman Coulter, US) particle size analyzer. All tests were performed in triplicate. The parameters of growth rate (G), nucleation rate (Bo) and suspension density (M_T) were determined from the number and size of crystals by the equations from previous studies [21-23] as stated in chapter 1. A plot of Ln (N) against crystal size (L) gives the slope and y-intercept for the determination of the Bo and G of melamine crystallization.

6.3.3 Optimization of MSMPR system for melamine crystallization

The system was optimized for melamine crystallization by testing for different percentages of melamine to cyanuric acid (0 – 100%). After the optimum ratio of melamine and cyanuric acid is found, the minimum concentration (0 – 10 mM) of melamine and cyanuric acid for melamine crystallization is investigated.

6.3.4 The physicochemical behaviour of melamine

The physiochemical changes in urinary pH and ionic strength on the effects of melamine crystallization were studied. The urinary pH was adjusted from 3.5 to 6.7

and the urinary ionic strength that affects the supersaturation of urine was adjusted with respect to sodium by adding (0.5 – 10)X of sodium chloride (BDH, Poole, England) for which 1X = 160 mM since urinary sodium can be between 15 – 250 mM per day depending on salt and hydration levels.

6.3.5 The interaction of melamine with other lithogenic salts

To study the effects of the presence of other lithogenic ions including calcium oxalate, calcium phosphate and uric acid on the melamine crystallization, the MSMPR was set up for (a) Calcium oxalate (CaOx) for which 5.77 mM calcium chloride (BDH, Poole, England) and 1.15 mM sodium oxalate (Sigma, St. Louis, US) were added separately into the chambers through addition of 2 inlet tubes; (b) (0.2 and 1)X of uric acid (UA) (BDH, Poole, England) were added into the test AU where 1X = 2.925 mM of uric acid; (c) (0.2 – 1)X of calcium phosphate (CaP) were added into the test AU where 1X = 4 mM calcium chloride (BDH, Poole, England) and 25 mM disodium hydrogen phosphate (BDH, Poole, England).

6.3.6 Melamine response to urinary tract infection (UTI)

MSMPR system to mimic UTI was set up by addition of *Escherichia coli* (*E. coli*) (ATCC 25922) into test AU. A 1.5 x 10^8 colony forming units (CFU)/mL stock *E. coli* suspension was freshly prepared by suspending of *E. coli* in normal saline to 0.5 McFarland turbidity standard (Key Scientific, Texas, USA) that had 0.132 absorbance

at 600 nm measured by spectrophotometer. Clinically associated (infective) dosage of *E. coli* (1 x 10^5 CFU/mL of urine) and 10 times lower and higher dosages were used by diluting the stock *E. coli* in test AU for this study.

6.3.7 Melamine response to the therapeutic agents

Known therapeutics used in the treatment of stone disease including potassium citrate and sodium bicarbonate were tested whether it will affect melamine crystallization. The MSMPR was setup for (a) (1 – 10)X of potassium citrate (Sigma, Steinheim, Germany) were added into the test AU where 1X = 2.17 mmol/L; (b) (12.5 – 50) mmol/L of sodium bicarbonate were prepared by diluting the 1000 mmol/L of sodium bicarbonate stock (8.4% w/v sodium bicarbonate intravenous infusion) solution (Braun, Melsungen, Germany) in test AU.

6.3.8 Human urine collection

This study is performed to investigate the effect of melamine cyanurate crystallization on human urines rather than AU. Early morning urine (EMU) was collected from 5 healthy subjects and urinalysis was performed on all samples to confirm that the subjects were free from urinary tract infection and bacteria contamination. Then, 300 mL of urine samples form each individual were centrifuged at 3000 g for 15 minutes to remove residual high density debris. The supernatants were then filtered though 0.45 μm pore size Millipore aseptic filtering system to remove

residual low-density cellular debris. The EMU after filtration were pooled for the optimization of MSMPR system for melamine crystallization on human urine.

6.3.9 Optimization of MSMPR system for melamine crystallization on human urine

The system was optimized for melamine crystallization in terms of crystallization kinetics on human urine by testing for different concentration (5, 10 and 15) mM of melamine and cyanuric acid in 1:1 ratio in human urine and compared with the established 5 mM of melamine and cyanuric acid in 1:1 ratio in AU as a control.

6.3.10 Shi Wei intervention study for melamine crystallization

Shi Wei (Folium Pyrrosiae) powder was brought from a local company with dosage specification applied in this study. Normal subjects were recruited with no known urological disorder history and other diseases (e.g. diabetics mellitus or hypertension with medications) that may affect the renal functions. Subjects had given informed consent to participant in this study and ethics approval was obtained from the Human Subjects Ethics Sub-committee of the Hong Kong Polytechnic University.

An intervention study was conducted with normal subjects taking 1 g dose of Shi Wei powder by dissolving the powder in 200 mL hot water twice per day (day and night after meal) for 1 week. EMU were collected before, 1 day and 1 week after the

Shi Wei intervention for melamine crystallization study by the MSMPR system. Urinalysis was performed on all samples to confirm that they were without evidence of urinary tract infection and confirm the integrity of the urine specimens (free of red blood cell, bacteria, protein and glucose). The effects of Shi Wei are reflected from the subject's urine as 'potential' for melamine crystallization.

6.3.11 Data and statistical analysis

The number and size of melamine crystals for each measurement were recorded and converted to the crystallization kinetics including the Bo, G, and M_T for analysis. GraphPad Prism version 4.03 for window (GraphPad Software, San Diego California, USA) was used to perform all statistical analysis in this experiment. The slope and y-intercept of the plot of Ln (N) against crystal sizes was tested by linear regression for each measurement. Linear plots were with high correlation coefficient of r^2 (>0.95), representing realistic growth and nucleation rates (absence of aggregation). One-way analysis of variance (ANOVA) was used for the comparison of the mean differences between concentrations of lithogenic ions, bacteria or therapeutic agents and their respective controls, if appropriate. A *P* value of less than 0.05 was considered a significant difference. All significant ANOVA test results were analysis by Dunnett's multiple comparisons post-test. The P value of less than 0.05, 0.01 and 0.001 were specified by the symbols *, **, and *** respectively in the graphs.

6.4 Results

6.4.1 Melamine crystallization kinetics analysis

The parameters of growth rate (G), nucleation rate (Bo) and suspension density (M_T) were determined from the number and size of crystals by the equations from previous studies [21-23] as stated in chapter 1. A plot of Ln (N) against crystal size (L) gives the slope and y-intercept (Figure 6.2) for the determination of the Bo and G of melamine crystallization.

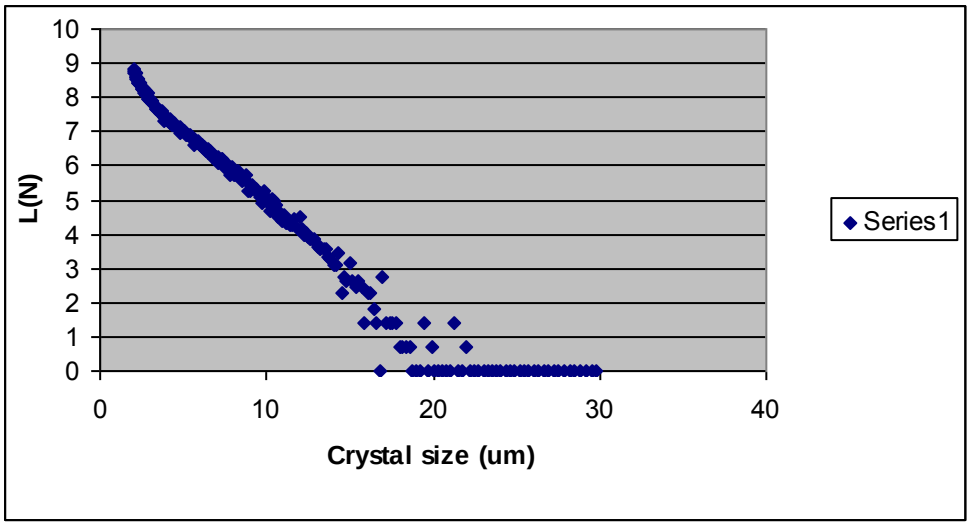
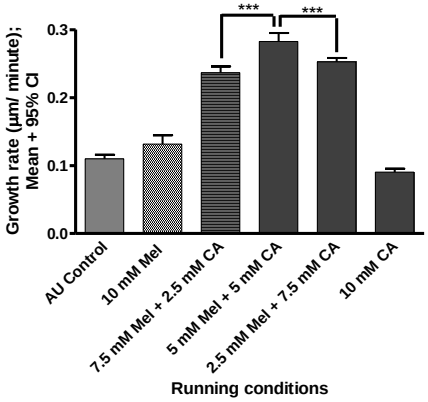


Figure 6.2. A plot of Ln (N) against crystal size (L) of melamine crystals gives the slope and y-intercept for the determination of the Bo and G of melamine crystallization.

6.4.2 Optimization of MSMPR system for melamine crystallization

The optimum ratio of melamine and cyanuric acid was found to be 1:1 (Figure 6.3). Melamine crystallization was concentration dependent and the minimum concentration that would trigger crystallization was at 5 mM (at 1:1 ratio), and at 10 mM it was 6-times as much (Figure 6.4).

A



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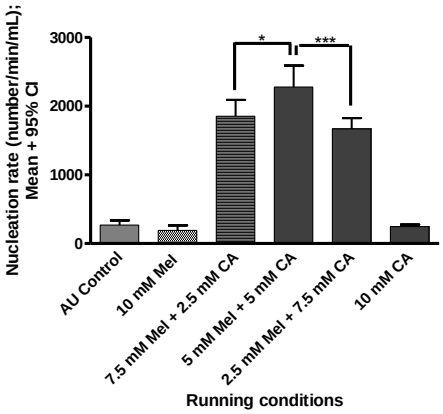

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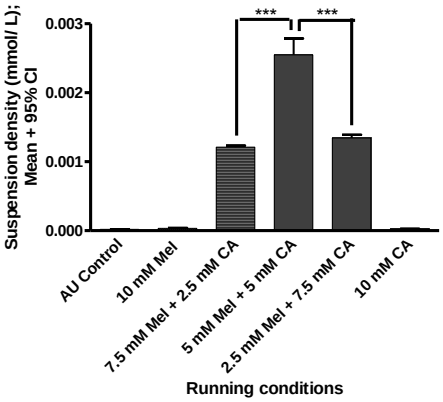


Figure 6.3. (A) Growth rates, (B) nucleation rates and (C) suspension densities (mean + 95% CI) of melamine (Mel) and cyanuric acid (CA). Different ratios of Mel and CA dissolved in MilliQ water was added to the test chamber with artificial urine (AU). AU was used for the Control chamber. (*P <0.05; ***P<0.001)

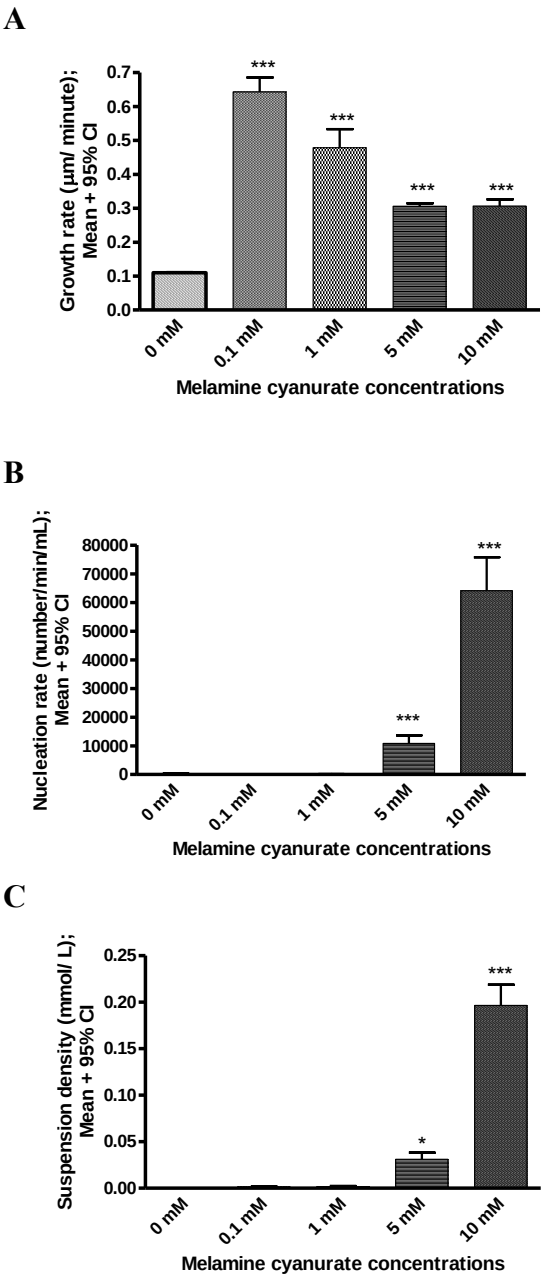


Figure 6.4. (A) Growth rates, (B) nucleation rates and (C) suspension densities (mean + 95% CI) of different concentrations of melamine cyanurate (ratio 1:1). Concentrations of Melamine cyanurate (1:1) from (0 – 10 mM) was dissolved in MilliQ water for test with AU. AU was used for the Control chamber. (*P <0.05; ***P<0.001 against 0mM control chamber)

6.4.3 Physicochemical aspects of melamine crystallization

At different pH (Fig. 6.5), melamine crystallized out from urine under normal urinary pH conditions (pH 5.0 – 6.5) but crystallization was strongly inhibited at pH 4.5 or lower. At pH higher than 6.7, calcium phosphate crystals start to precipitate and pH data beyond pH 6.7 would include both types of crystals. By varying the ionic strength based on sodium concentration, we observed no difference or trend on melamine crystallization (results not shown).

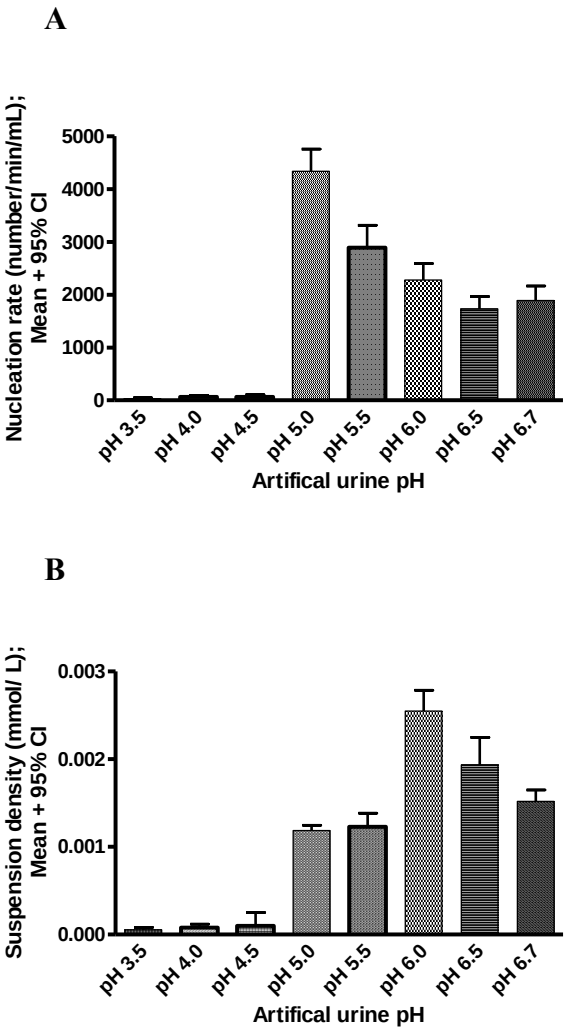


Figure 6.5. The effects of urinary pH on the (A) nucleation rates and (B) suspension densities (mean + 95% CI) of melamine cyanurate crystals. 5 mM of melamine and cyanuric acid is dissolved in MilliQ water for the Test with AU and AU for the Control. The pH of the artificial urine is prepared from phosphate buffer and adjusted with HCl to the desired pH.

6.4.4 Melamine crystallization with other endogenous urine factors

6.4.4.1 Melamine on calcium oxalate (CaOx) crystallization

The effects of melamine, cyanuric acid and melamine cyanurate on CaOx crystallization were profound (Figure 6.6). Melamine, cyanuric acid and melamine cyanurate were shown to enhance the nucleation rate (Bo) and suspension density (M_T) together with reduced growth rate (G), implicating that they lead to the formation of more smaller CaOx crystals as observed microscopically. This suggested that all of them promoted the CaOx crystallization. Cyanuric acid was a stronger promoter than melamine for CaOx crystallization since the changes in the Bo and G as well as M_T were more pronounced in cyanuric acid. The effects of melamine cyanurate in the promotion of CaOx crystallization were much higher when compared with melamine and cyanuric acid alone at 5 and 10 mM concentrations.

However, as little as 0.1 mM of melamine can enhance the CaOx precipitation and significant changes in Bo and M_T when compared to the crystallization of CaOx alone (Figure 6.6). This is an important finding in that while it requires higher concentrations of cyanuric acid (1 mM) and melamine cyanurate (5 mM) to promote the precipitation of CaOx, only 0.1 mM of melamine present in the urine will increase the precipitation of CaOx.

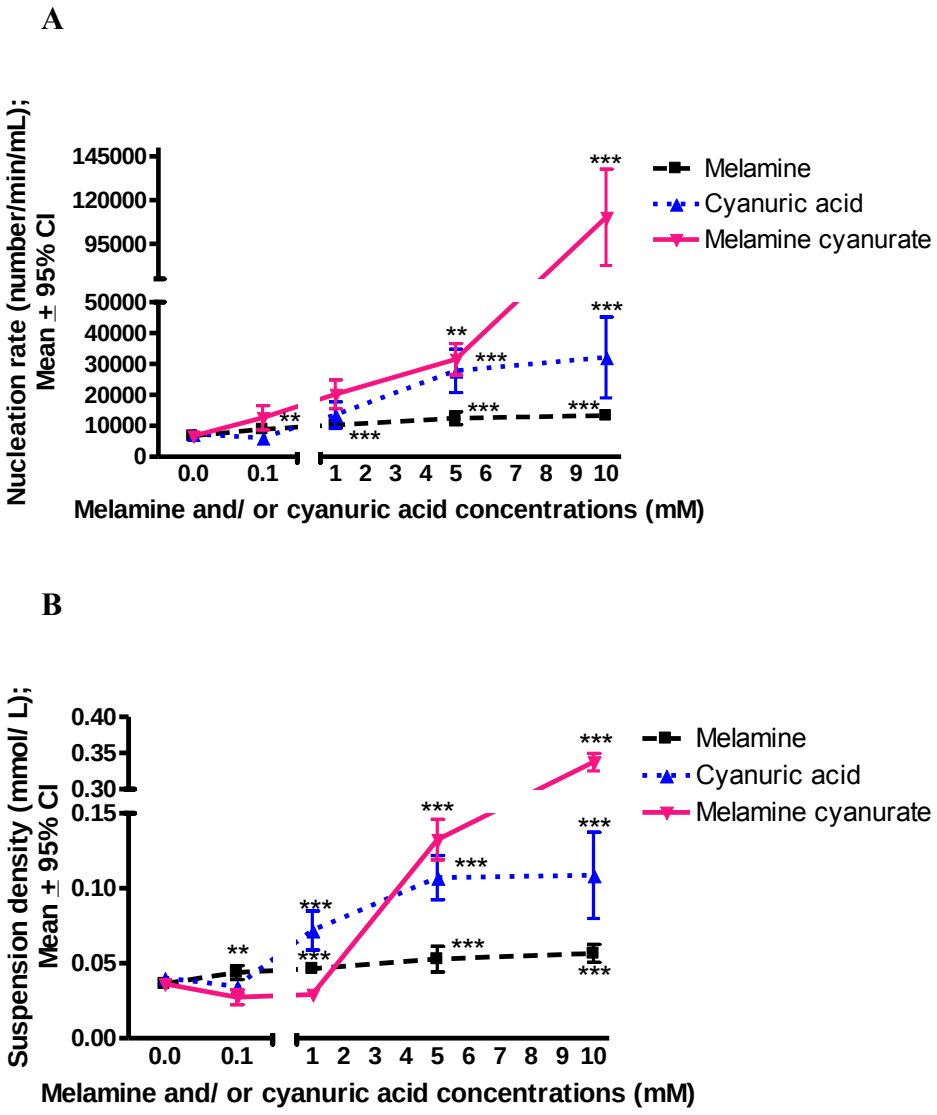


Figure 6.6. The effects of different concentrations of melamine, cyanuric acid, and melamine cyanurate on the (A) nucleation rates and (B) suspension densities of calcium oxalate crystals. 5mM of each constituent was dissolved in MilliQ and added into the test chamber with AU while AU only for control. $CaCl_2$ (5.77mM) and $Na_2Oxalate$ (1.15 mM) were fed to the Test and Control crystallizer separately to induce calcium oxalate crystallization in the respective chambers. (**P<0.01; ***P<0.001 against 0mM control chamber)

6.4.4.2 Uric acid (UA) on melamine crystallization

Our findings showed that the nucleation rates and suspension densities of melamine crystallization were significantly reduced by addition of UA (Figure 6.7) implicating that the melamine cyanurate crystallization was inhibited by UA.

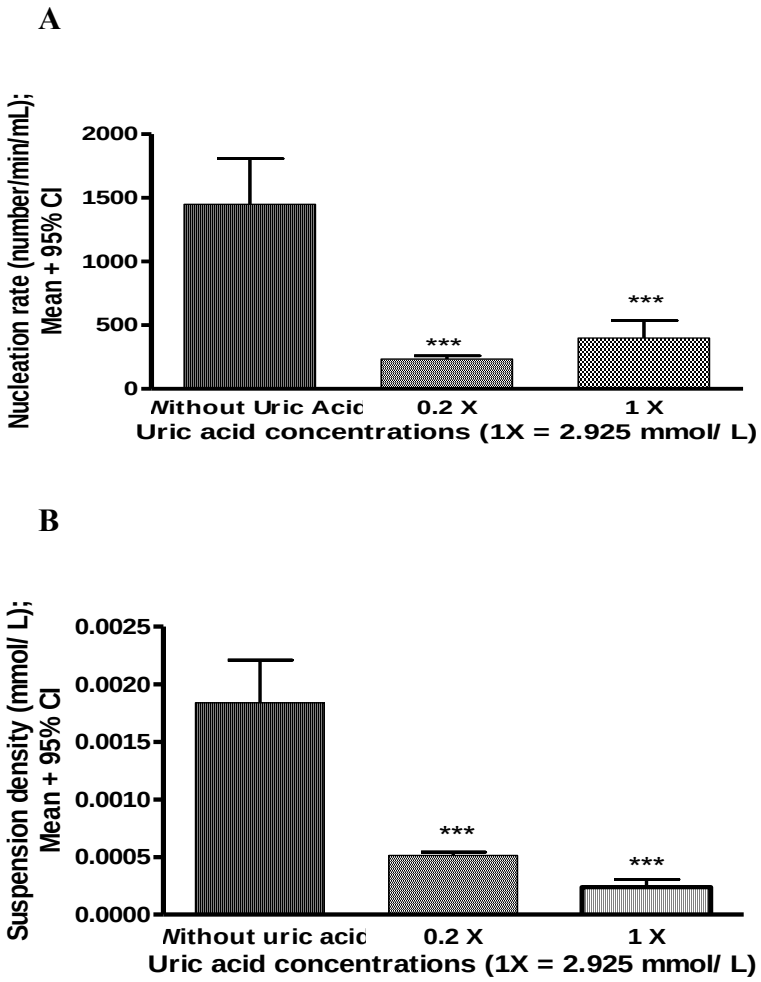
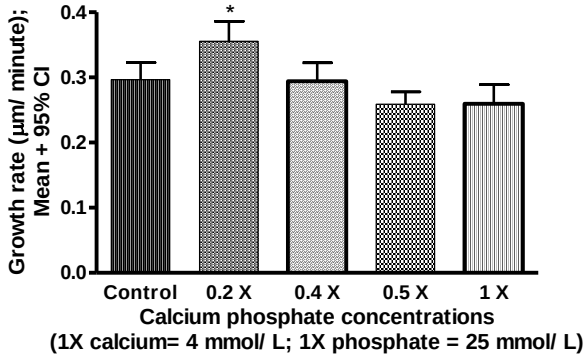


Figure 6.7. Inhibitory effect of uric acid on the (A) nucleation rates and (B) suspension densities (mean + 95% CI) of melamine cyanurate crystals. Uric acid (1X = 2.925 M & 0.2X) was added to test AU or AU (Control) and 5mM melamine and cyanuric acid were fed to the Test and control chambers. (***P<0.001 against control chamber containing no uric acid)

6.4.4.3 Calcium phosphate (CaP) on melamine crystallization

However, the addition of CaP into the urine did not give a profound result on melamine crystallization. The nucleation rates of melamine crystals was enhanced significantly at 1X CaP concentration only (Figure 6.8) but no trends were observed on the growth rates and suspension densities of melamine.

A



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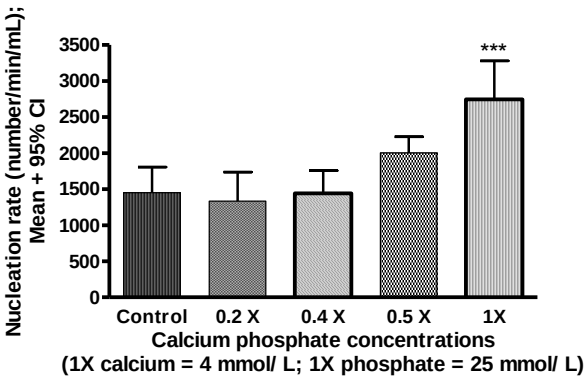


Figure 6.8. The effects of calcium phosphate (CaP) on the (A) growth rates and (B) nucleation rates (mean + 95% CI) of melamine cyanurate crystals. AU was used as control. Appropriate concentrations of calcium and phosphate were fed to the test chambers separately to induce calcium phosphate crystallization. (*P<0.05; ***P<0.001 against control chamber containing no calcium phosphate)

6.4.4.4 Urinary tract infection (UTI) on melamine crystallization

The presence of *Escherichia coli* (*E. coli*) mimics urinary tract infection and reduced the nucleation rates, but enhanced the growth rates and suspension densities (Figure 6.9) indicating the promotion of melamine cyanurate crystallization. Microscopic examination showed that the melamine crystals were larger.

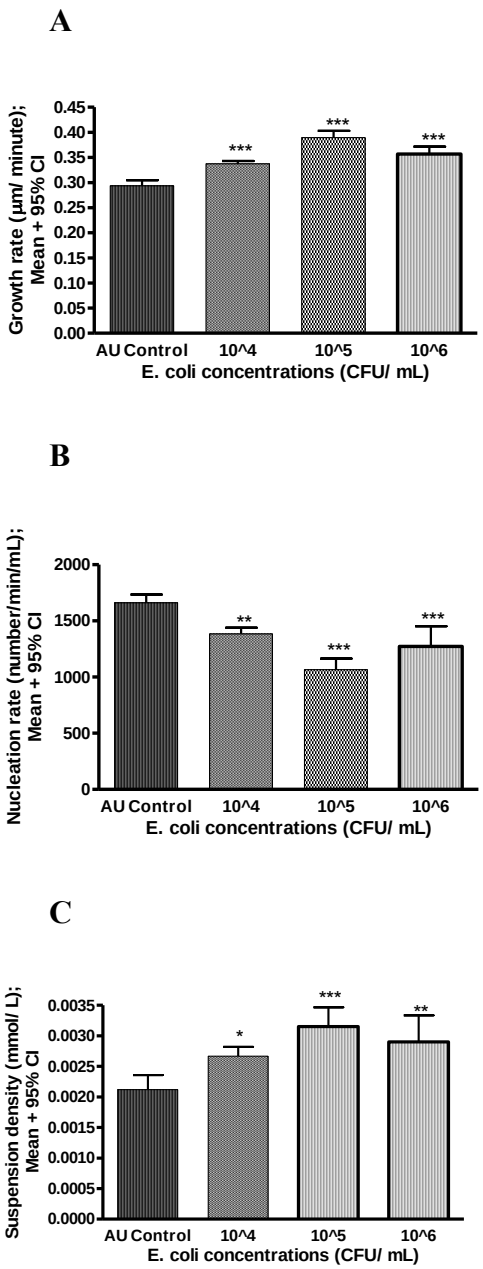


Figure 6.9. The effects of the presence of *E. Coli* in urine on the (A) growth rates, (B) nucleation rates and (C) suspension densities (mean + 95% CI) of melamine cyanurate crystals. Clinically relevant dosage of E. coli (1 x 10^5 colony forming unit/mL of urine) was added to the test AU or AU (Control) and 5mM melamine and cyanuric acid were fed to the test and control chambers. (*P<0.05; **P<0.01; ***P<0.001 against control chamber)

6.4.5 Melamine response to the therapeutic agents

6.4.5.1 Melamine response to potassium citrate

Increasing citrate had a significant decrease in nucleation rates and suspension densities but no effect on growth rates (Figure 6.10) of melamine crystals suggesting that citrate inhibited melamine crystallization through chelation of other ions.

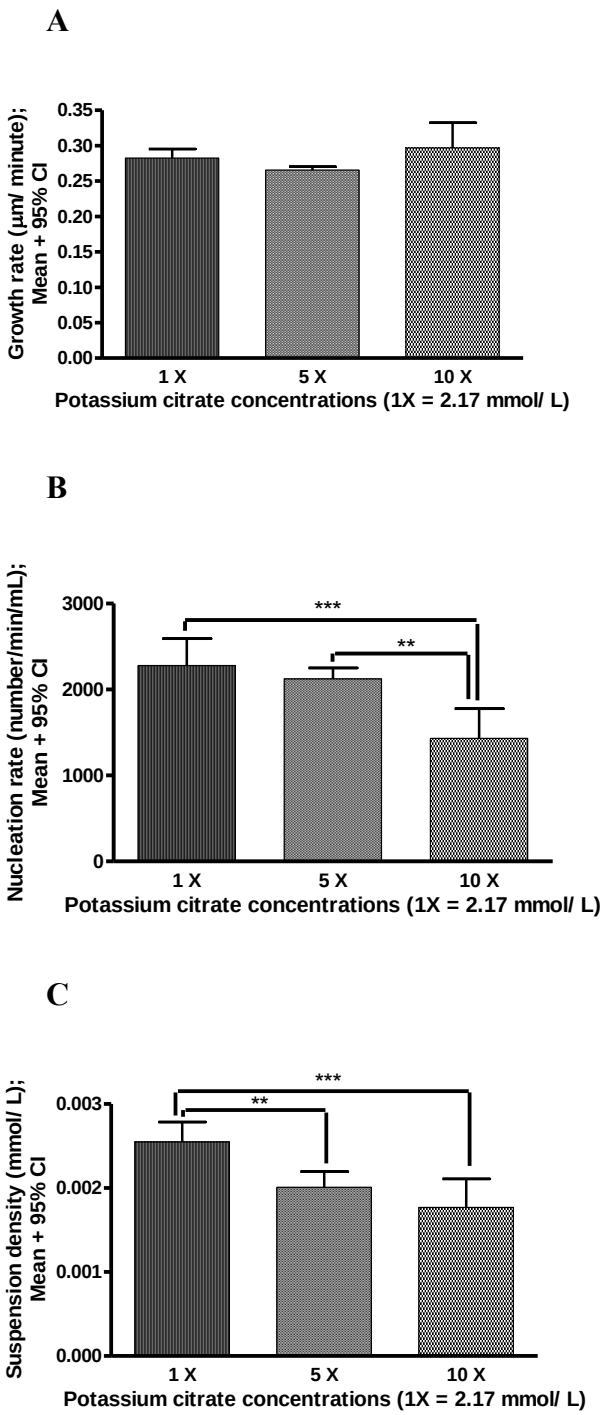


Figure 6.10. The effects of citrate on the (A) growth rates, (B) nucleation rates and (C) suspension densities (mean + 95% CI) of melamine cyanurate crystals. The relevant concentration of citrate was added to the AU and AU as control and 5mM melamine and cyanuric acid were fed to the test and control chambers. (**P<0.01; ***P<0.001)

6.4.5.2 Melamine response to sodium bicarbonate

For bicarbonate, increasing concentration brought about significant increase in nucleation rates, with reduced growth rates and suspension densities (Figure 6.11) of melamine crystals suggesting that bicarbonate inhibited melamine cyanurate crystallization by formation of smaller melamine crystals.

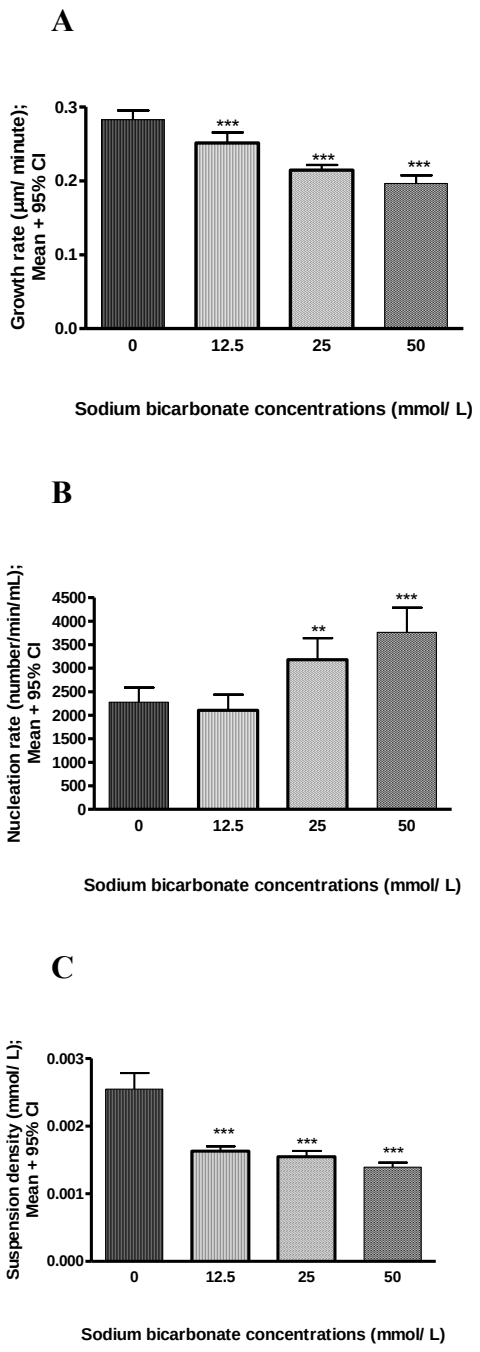
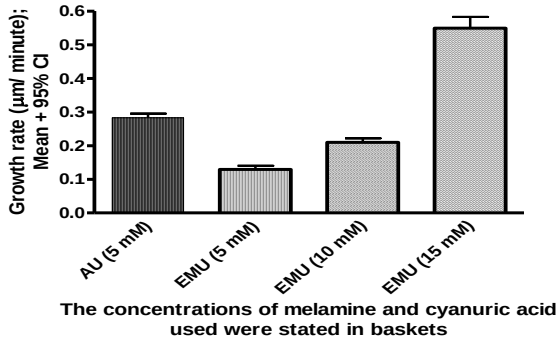


Figure 6.11. The effects of bicarbonate on the (A) growth rates, (B) nucleation rates and (C) suspension densities (mean ± 95% CI) of melamine cyanurate crystals. The relevant concentration of bicarbonate was added to the AU or AU as control and 5mM melamine and cyanuric acid were fed to the test and control chambers. (**P<0.01; ***P<0.001 against 0mM control)

6.4.6 Effects of human early morning urine (EMU) on melamine crystallization

The results (Figure 6.12) found that there was an inhibition in the suspension densities, nucleation rates and growth rates of melamine cyanurate crystals at 5 and 10 mM concentrations in human EMU when compared with 5 mM concentration of melamine cyanurate in AU. Thus, the optimized concentrations of melamine and cyanuric acid for human urinary melamine crystallization is found to be at 15 mM.

A



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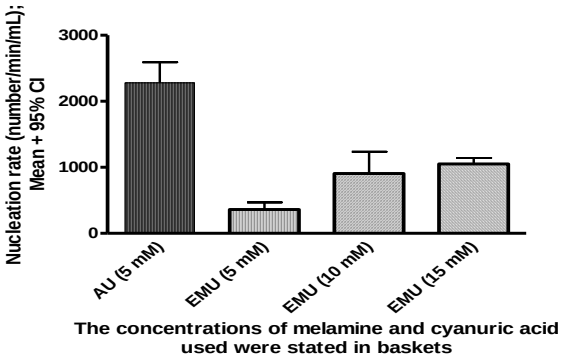

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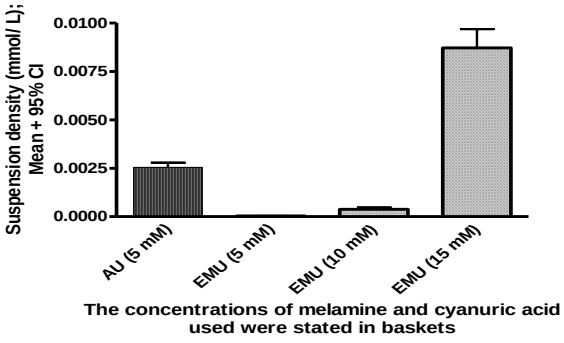
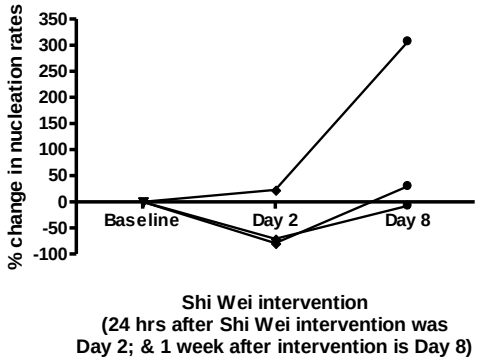


Figure 6.12. The (A) growth rates, (B) nucleation rates and (C) suspension densities (mean + 95% CI) of melamine cyanurate in 1:1 ratio of different concentrations of melamine and cyanuric acid in human early morning urine (EMU) and 5 mM of melamine and cyanuric acid in 1:1 ratio in artificial urine (AU) as a control.

6.4.7 Effects of Shi Wei intervention on melamine crystallization

Shi Wei showed a quick effect (1 day after intervention) for significant reduction of the suspension densities of melamine cyanurate crystallization (Figure 6.13). However, the inhibitory effects of Shi Wei on melamine cyanurate crystallization subsided for subjects taking Shi Wei supplementation for over 1 week.

A



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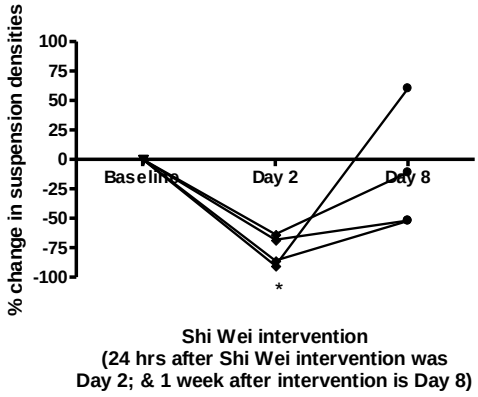

Figure 6.13. The effects of subject's human urines on the changes (%) of (A) nucleation rates and (B) suspension densities of melamine cyanurate crystallization before and after Shi Wei intervention. (*P<0.05 against baseline)

6.5 Discussion

We began to query some fundamental questions as to what is the optimal proportion of melamine and cyanurate, at what pH and ionic strength would crystallization occur? That is, does urine provide an optimal condition for melamine crystallization?

By studying various proportions of melamine: cyanurate, melamine crystallization was maximal for nucleation rate (Bo), growth rate (G) and suspension density (M_T) at 1:1 ratio (Figure 6.3). Previous studies showed that cyanuric acid and melamine complex was formed directly by simultaneous deposition of cyanuric acid and melamine [24]. A very heat stable solid 1:1 melamine-cyanuric acid complex was formed by hydrogen bonding after mixing melamine and cyanuric acid in aqueous solution [25]. This leads to the formation of large, stable structurally defined aggregates at equilibrium [25]. The process for the formation of cyanuric acid-melamine lattice is called 'molecular self-assembly' that can be applied for synthesis of nanostructures [25]. Both melamine and cyanuric acid alone were shown to have little crystallization and no statistical significant different from the AU as demonstrated from the growth and nucleation rates. This suggested that the crystallization of melamine required the presence of cyanurate, which is also supported by other studies [26-28]. As cyanurate is usually present as a contaminant in melamine only [29], the concentration of cyanurate is not very high. This may explain the overall incidence of stone detected in these children is still not very high. From figure 6.4, we observed that

melamine crystallization is concentration dependent and the minimum concentration that would trigger crystallization was at 5mM (at 1:1 ratio), and at 10mM it is 6-times as much. Therefore, fetus and younger children are more vulnerable to the formation of melamine stones due to the relative higher intake of melamine-tainted milk product [30].

Therapeutic agents aimed at preventing kidney stones by alkalization or acidification of urine may also be used for patients with melamine stones since melamine crystallized out from AU under acidic pH (Figure 6.5) but the crystallization was strongly inhibited at pH 4.5 or lower. To avoid the formation of uric acid (UA) in acidic urine and calcium phosphate (CaP) under alkaline urine after the treatments, it is suggested that the patients should drink more water and the treatment should include diuretics for flushing out the crystals and diluting the lithogenic ions.

As the urine is a complex liquid containing various ions and the composition and ionic strength may vary, the effects of ionic strength keeping the ratio at 1:1 and pH at 6.0 were also investigated. The non-significant results of changing urinary ionic strength on melamine crystallization can be attributed to the fact that urine itself is supersaturated with other lithogenic ions and any change in ionic strength is subtle.

Even in low melamine consumption subjects, they may also have an increased chance for calcium oxalate (CaOx) stone formation as 0.1 mM of melamine in urine can enhance the CaOx precipitation in our results (Figure 6.6). This correlates with

another study that even exposure to low dose of melamine will have higher chance to develop both UA and calcium urolithiasis in adults [31]. As a result, it may still be worthwhile to screen older children and even adult for renal stones after melamine exposure.

Structurally, melamine and cyanurate share very good epitaxial relationship with the endogenous UA found in human urine. This suggests that given the correct pH and supersaturation, the precipitation of any one of these ions will cause precipitation of the other. Since the optimal melamine precipitation was in the same pH region as UA, it is not surprising that UA also precipitates out by epitaxy. Thus, UA can actually interact with melamine crystallization by co-precipitate with melamine so that the melamine cyanurate crystallization was inhibited (Figure 6.7). Cyanuric acid is an inhibitor of hepatic UA oxidase that leads to the enhancement of serum UA levels [32]. Excessive UA excreted in the renal tubules can compete with cyanuric acid for melamine binding. Clinicians did observe that UA was co-precipitated with melamine in infants and young children [33] in a molar ratio of 1.2 – 2.1 of UA to 1 of melamine.

However, the addition of calcium phosphate (CaP) into the urine did not give a profound result on melamine crystallization (Figure 6.8) and thus it is not suggestive that CaP has interaction with melamine crystallization.

Urinary tract infection (UTI) is common in children [16] and it may interacts with melamine crystallization in infants and child. The presence of *E. coli*, which is the

main UTI causative bacteria [18], promoted melamine crystallization (Figure 6.9) and larger crystals were observed under microscopic examination. This may encourage the blockage of the narrow urinary tract. Hence, it is recommended to treat active UTI to reduce risk of crystal formation.

Current therapeutics for urolithiasis includes drugs that chelate lithogenic ions, alkalize or acidify urine pH to prevent precipitation and maybe given with diuretics. Two clinically relevant therapeutics, potassium citrate and sodium bicarbonate which are also administrated to children with melamine stones were studied. Both of them showed reduction of melamine crystallization (Figure 6.10 and 6.11) through different mechanisms. Increasing citrate reduced the overall supersaturation of urine to inhibit crystallization through chelation of other cations (e.g. calcium and magnesium). Bicarbonate inhibited melamine crystallization by formation of smaller melamine crystal. In biological crystallization, this is also a strategy to reduce overall supersaturation, to nucleate numerous small crystals but at the same time inhibiting growth. This will allow the crystals, small enough to pass in the urinary tract without obstruction.

A traditional Chinese herbal medicine – Shi Wei was chosen for an intervention study regarding its effects on melamine crystallization. MSMPR system was optimized for the determination of melamine crystallization by human urine instead of AU before the start of this experiment. The results showed that melamine crystallization was inhibited at 5 and 10 mM of melamine and cyanuric acid (Figure 6.12) in human early

morning urine (EMU) and this may be probably due to EMU containing urinary macro-molecules (UMMs) such as glycosaminoglycans and glycoproteins which can inhibit melamine crystallization. Thus, higher concentrations of melamine and cyanuric acid (15 mM) were needed to induce optimal melamine crystallization for kinetics studies of suspension densities, nucleation and growth rates.

Traditional Chinese medicines (TCM) has been used for treating renal stones due to their anti-lithogenic activities [34]. Previous study investigated different types of Chinese herbal medicine used by Chinese practitioners for the treatment of renal stones and the results showed that all herbs can inhibit the CaOx crystallization by promoting the nucleation while inhibiting the growth of CaOx crystals [35]. Shi Wei was one of the most potent herb for inhibition of crystallization and was chosen for this intervention study. It showed a quick effect (1 day after intervention) for the significant reduction of melamine crystallization (Figure 6.13) and this is correlated with another previous study that Shi Wei has a potential to inhibit urinary crystallization by reducing the urinary specific gravity and enhancing the urinary magnesium. Reducing the specific gravity of urine reflected the reduction of urinary supersaturation. Enhancement of the urinary magnesium, which is an inhibitor of crystallization, can form soluble ion complexes with lithogenic anions to reduce the potential of crystallization, for example, magnesium oxalate being more soluble than calcium oxalate.

However, the inhibitory effects of Shi Wei on melamine crystallization subsided for subjects taking 1 week Shi Wei supplementation (Figure 6.13). In Chinese herbal medicines, herbs are used for maintaining people's health and homeostasis between 'yin' and 'yang' for which they are referred to 'cold' and 'warm' in nature, respectively. Thus, herbal supplementation can adjust the balance of mineral electrolytes in order to maintain the urinary system to the equilibrium state through different kinds of feedback systems such as the endocrine system [36,37].

Conclusion

Based on the results, a treatment plan for melamine exposure is proposed for patients with acute melamine exposure. Besides cessation of melamine intake, potassium citrate and sodium bicarbonate can be applied clinically with good efficacy in children [38,39] with melamine stones. To avoid the formation of CaOx and CaP at neutral and alkaline urine after the treatments, adequate hydration can help to reduce the urinary concentration of melamine and thus melamine crystal and CaOx and CaP crystal formation. The treatment should also include diuretics for flushing out the crystals and diluting the urine and lithogenic ions as well. Shi Wei can act as supportive treatment for quick relief of further melamine stone formation.

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Chapter 7: Conclusions and suggestions for future research

Since renal stone formation starts from crystallization of lithogenic salts, studying *in vitro* crystallization can provide fundamental information to urolithiasis research. There are many different methods developed for qualitative and/or quantitative measurement of *in vitro* crystallization activity. Mixed suspension mixed product removal (MSMPR) system aims to mimic the continuous flow urinary system for which a continuous input of reactants (lithogenic ions) in a supersaturated solution such as artificial urine (AU) and removal of mixed products allows for the saturation in the system to remain constant. This unique system can allow the measurement of growth and nucleation rates as well as suspension densities of crystals at the same time independently for the investigation of urinary factors that affect the kinetics on crystallization in this study.

Urinary glycoproteins (GPs) and urinary glycosaminoglycans (GAGs) are two major urinary macromolecules that can act as modifiers on crystallization. Different studies based on a variety of methods have reported the effects of urinary GPs and GAGs on the calcium oxalate (CaOx) crystallization, that may confound the findings and lead to inconclusive results. Besides, all studies were concentrated on one or two individuals GPs or GAGs on CaOx but there are no studies concerning native urinary GPs or GAGs on CaOx crystallization.

Further studies are needed to look into the composition of GPs in the urine that contributed to the promotory effects and also the expression and regulation of these GPs due to the complexities of the abundance, varieties, and nature of proteins present.

Total urinary GAGs showed no statistically significant effect on CaOx crystallization and this was different from other studies that showed GAGs do inhibit the CaOx crystallization. This may be explained by the disparate effects (promotory and inhibitory) on individual GAGs that neutralized their effects on crystallization. Therefore, individual GAGs were studied and this showed GAGs subpopulations had diverse effects on CaOx crystallization. The mechanism by which individual GAGs influence the crystallization process is still unknown and it is worthy to investigate the function of individual GAG in the CaOx crystallization in terms of its chemical structural difference (e.g. its peptide content, length of the carbohydrate-peptide linkage region, degree of sulphation, or its molecular size) between different kinds of GAGs.

Urinary excretion of GAGs was significantly lower in stone formers (SF) than in normal individuals. However, there are no systematic studies concerning urinary GAG excretion and sub-GAG species such as HA in stone-formers after stone removal by extracorporeal shock wave lithotripsy (post-ESWL) that may affect the risk for the recurrence of renal stones.

Active-SF and post-treated SF groups had lower total GAGs content but increased proportion of HA than that of the normal controls indicating that urinary GAGs and HA are probably protective and risk factors respectively for the occurrence and recurrence of renal stone disease. The increased production of GAGs by renal tubular epithelial cells protect cells from binding of CaOx crystals to prevent CaOx stone formation while HA is a non-sulfated GAG with crystallization-promoting activity. It is therefore suggested that HA becomes an accidental participant in the pathogenesis of stone disease and patients who have undergone procedures or treatments, may have compromised the integrity of the urothelium lining with subsequent HA being released as an inflammatory response molecule. This release of HA can be a potential diagnostic marker though further studies are needed to look into this.

Melamine renal stone disease appeared in Chinese infants in late 2007 lead to the concern as to how melamine affected renal stone disease. Unlike CaOx crystallization, the physicochemical nature of melamine in urine and its handling were largely unknown at that time.

Melamine crystallization was maximal at 1:1 ratio by studying various proportions of melamine: cyanurate. Melamine crystallizes in normal urinary pH (pH 6.0) so that normal urine can provide an optimal condition for melamine crystallization. However, melamine crystallization was strongly inhibited at pH 4.5 or lower. Therefore, therapeutic agents aimed at preventing renal stones by alkalization or acidification of urine may also be used for patients with melamine stones.

Melamine can interact with other lithogenic salts including calcium, oxalate and uric acid (UA). This is correlated with another study that exposure at low dose of melamine will have higher chance to develop both UA and calcium urolithiasis in adults. As a result, it may still be worthwhile to screen older children and even adult for renal stones after melamine exposure.

The presence of UA significantly reduced melamine crystallization. Since the optimal melamine precipitation was in the same pH region as uric acid, it is not surprising that uric acid also precipitates out. Structurally, melamine and cyanurate share very good epitaxial relationship with the endogenous UA found in human urine. This suggests that given the correct pH and supersaturation, the precipitation of any one these ions will cause precipitation of the other. Thus, UA can actually interact with melamine crystallization by co-precipitating with melamine so that the melamine cyanurate crystallization was inhibited. This is correlated with study that UA was co-precipitated with melamine in infants and young children as observed by the clinicians. Further study of melamine crystallization with uric acid should be more clinical relevant as uric acid is usually excreted in urine.

Currently used therapeutic agents including potassium citrate and sodium bicarbonate on CaOx stone disease were also prescribed on melamine stone subjects. However, the effectiveness of these drugs on melamine crystallization was still unknown. Both citrate and bicarbonate did significantly reduce melamine crystallization in this study through different mechanisms. Citrate is thought to be

through chelation of other cations (e.g. calcium and magnesium), reducing the overall supersaturation of urine to inhibit crystallization. Bicarbonate inhibited melamine crystallization by formation of smaller melamine crystals. In biological crystallization, this is also a strategy to reduce overall supersaturation, to nucleate numerous small crystals but at the same time inhibiting growth. The results from this study indicated that currently used therapeutic agents are effective against melamine crystallization and melamine stone formation.

Traditional Chinese medicines (TCM) have been used for treating renal stones due to their anti-lithogenic activities. Previous study found that a Chinese herb Shi Wei (Folium Pyrrosiae) has a potential to inhibit urinary crystallization by reducing the urinary specific gravity and enhancing the urinary magnesium. This study showed that the human urine after Shi Wei supplementation had significant acute inhibitory effect on melamine crystallization but the effect was diminished or even levelled off after 1 week supplementation. Thus, Shi Wei may be a suitable short-term therapeutic agent for the quick control of melamine stones in infants by inhibition of further melamine crystals formation.

A treatment plan for melamine exposure is proposed for patients with acute melamine exposure. Besides cessation of melamine intake, potassium citrate and sodium bicarbonate can be applied clinically with good efficacy in children with melamine stones. To avoid the formation of CaOx and calcium phosphate (CaP) at neutral and alkaline urine after the treatments, adequate hydration can help to reduce

the urinary concentration of melamine and thus melamine crystal and CaOx and CaP crystal formation. The treatment should also include diuretics for flushing out the crystals and diluting the urine and lithogenic ions as well. Traditional Chinese medicines such as Shi Wei can act as supportive treatment for quick relief of further melamine stone formation.

Apart from the knowledge of melamine in an *in vitro* crystallization achieved in this study, further study can be done on the mechanisms of melamine crystals-cell interactions such as how intestinal cells uptake and transport of melamine? how renal cells deposit melamine? will melamine induced oxidative stress and injury to renal cells? what are the sequent inflammatory responses and gene expressions of the injured renal cells in response to melamine? Future studies would involve *in vitro* cell culture model as well as *in vivo* animal model to extrapolate these laboratory studies.

Appendices

Information sheet and consent form (English and Chinese versions) for recruitment of stone-formers (SF), post-treated SF, and normal individuals and early morning urine collection.

Information sheet and consent form (English and Chinese versions) for recruitment of healthy subjects for Shi Wei intervention study and early morning urine collection.

THE HONG KONG
POLYTECHNIC UNIVERSITY

香港理工大學
護理學院
School of Nursing

香港 九龍 紅磡
Hung Hom Kowloon Hong Kong

INFORMATION SHEET

(Systematic study to delineate diagnostic markers for idiopathic stone formers)

You are invited to participate in a study conducted by Dr. Danny Gohel and his research team of the School of Nursing in The Hong Kong Polytechnic University.

The aim of this study is detect diagnostic markers of renal stone occurrences.

The testing should not result in any undue discomfort, but you will need to collect urine in bottles.

You have every right to withdraw from the study before or during the measurement without penalty of any kind. All information related to you will remain confidential, and will be identifiable by codes known only to the researcher.

If you have any complaints about the conduct of this research study, please do not hesitate to contact Mr. Eric Chan, Secretary of the Human Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University in person or in writing (c/o Human Resources Office in Room M1303 of the University).

If you would like more information about this study, please contact Dr. Danny Gohel at tel. no. 2766 7883 .

Thank you for your interest in participating in this study.

Poon Ngork Wah
Investigator

Dr. Danny Gohel
Principal Investigator

THE HONG KONG
POLYTECHNIC UNIVERSITY
香港理工大學
護理學院
School of Nursing

香港 九龍 紅磡
Hung Hom Kowloon Hong Kong

有關資料

(突發性腎結石患者診斷指標的系統性研究)

誠邀閣下參加由香港理工大學護理學院高文宇博士及其研究小組負責執行的研究計劃 。

這項研究的目的是尋找腎結石產生的診斷指標。

這項研究並不會為閣下帶來任何不適，但閣下需要用護士所給予的瓶子來收集尿液。

閣下享有充分的權利在研究開始之前或之後決定退出這項研究, 而不會受到任何對閣下不正常的代遇或 責任追究。 凡有關閣下的資料均會保密， 一切資料的編碼只有研究人員知道。

如果閣下有任何對這項研究的不滿， 請隨時與香港理工大學人事倫理委員會秘書陳先生親自或寫信聯絡 (地址: 香港理工大學人力資源辦公室 M1303 室轉交) 。

如果閣下想獲得更多有關這項研究的資料， 請與高文宇博士聯絡， 電話: 3400 8584。

謝謝閣下有興趣參與這項研究 。

研究員
潘岳華

主研究員
高文宇博士

THE HONG KONG
POLYTECHNIC UNIVERSITY
香港理工大學
護理學院
School of Nursing

香港 九龍 紅磡
Hung Hom Kowloon Hong Kong

CONSENT TO PARTICIPATE IN RESEARCH

I _____ hereby consent to participate in the captioned research conducted by Dr. Danny Gohel and his research team at the School of Nursing, The Hong Kong Polytechnic University.

I understand that information obtained from this research may be used in future research and published. However, my right to privacy will be retained, i.e., my personal details will not be revealed.

The procedure as set out in the attached information sheet has been fully explained. I understand the benefits and risks involved. My participation in the project is voluntary.

I acknowledge that I have the right to question any part of the procedure and can withdraw at any time without penalty of any kind.

Name of participant _____

Signature of participant _____

Name of Parent or Guardian (if applicable) _____

Signature of Parent or Guardian (if applicable) _____

Name of researcher _____

Signature of researcher _____

Date _____

THE HONG KONG
POLYTECHNIC UNIVERSITY
香港理工大學
護理學院
School of Nursing

香港 九龍 紅磡
Hung Hom Kowloon Hong Kong

參與研究同意書

本人_____同意參加由香港理工大學護理學院高文宇博士及其研究小組負責執行的研究項目。

我理解此研究所獲得的資料可用於未來的研究和學術交流。 然而我有權保護自己的隱私， 我的個人資料將不能洩漏。

我對所附資料的有關步驟已經得到充分的解釋。 我理解可能會出現的風險。 我是自願參與這項研究。

我理解我有權在研究過程中提出問題, 并在任何時候決定退出研究而不會受到任何不正常的待遇或責任追究。

參加者姓名: _____

參加者簽名: _____

父母姓名或監護人姓名: (如需要) _____

父母或監護人簽名: (如需要) _____

研究人員姓名: _____

研究人員簽字: _____

日期: _____

The Hong Kong Polytechnic University

Department of Health Technology & Informatics

INFORMATION SHEET

Project title: Effect of Traditional Chinese Medicine on human urine crystallization properties of melamine cyanurate crystals

You are invited to participate in a study conducted by **Ngork Wah Poon**, who is research assistant of the Department of Health Technology & Informatics in The Hong Kong Polytechnic University.

You need to take the a Chinese herb power twice per day for 7 consecutive days. Also, you require to leave early morning urine and blood samples before, 1 day and 8 days after treatment for urine and blood chemistry analysis.

You have every right to withdraw from the study before or during the measurement without penalty of any kind. All information related to you will remain confidential, and will be identificable by codes known only to the researcher.

If you have any complaints about the conduct of this research study, please do not hesitate to contact Mr. Eric Chan, Secretary of the Human Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University in person or in writing (c/o Human Resources Office in Room M1303 of the University).

If you would like more information about this study, please contact **Ngork Wah Poon** at tel. no. **9780** or his supervisor **Dr. Mayur Danny Indulal GOHEL** at tel. no. **3400 8584**

Thank you for your interest in participating in this study.

Name of investigator

Dr. Danny I Gohel
Ngork Wah Poon

香 港 理 工 大 學

醫 療 科 技 及 資 訊 學 系

有 關 資 料

誠 邀 閣 下 參 加 Ngork Wah Poon 負 責 執 行 的 研 究 計 劃。 她 是 香 港 理 工 大 學 醫 療 科 技 及 資 訊 學 系 學 生。

這 項 研 究 的 目 的 是 **中 醫 中 藥 對 腎 石 症 的 功 效**。閣 下只 需 按 照 指 示，連 續 服 用 給 予 的 單 方 中 藥 七 天，而 且 在 療 程 前 和 完 成 療 程 後 留 下 小 便，用 作 研 究。

閣 下 享 有 充 分 的 權 利 在 研 究 開 始 之 前 或 之 後 決 定 退 出 這 項 研 究, 而 不 會 受 到 任 何 對 閣 下 不 正 常 的 代 遇 或 責 任 追 究 。凡 有 關 閣 下 的 資 料 均 會 保 密, 一 切 資 料 的 編 碼 只 有 研 究 人 員 知 道。

如 果 閣 下 有 任 何 對 這 項 研 究 的 不 滿, 請 隨 時 與 香 港 理 工 大 學 人 事 倫 理 委 員 會 秘 書 親 自 或 寫 信 聯 絡 (地 址：香 港 理 工 大 學 人 力 資 源 辦 公 室 M1303 室 轉 交)。

如 果 閣 下 想 獲 得 更 多 有 關 這 項 研 究 的 資 料, 請 與 Ngork Wah Poon 聯絡, 電 話 9780 或 接 觸 她 的 導 師 **Dr. Mayur Danny Indulal GOHEL,** 電 話 3400 8584

謝 謝 閣 下 有 興 趣 參 與 這 項 研 究。

研 究 員

Dr. Danny I Gohel
Ngork Wah Poon

The Hong Kong Polytechnic University

Department of Health Technology & Informatics

CONSENT TO PARTICIPATE IN RESEARCH

Project title: Effect of Traditional Chinese Medicine on human urine crystallization properties of melamine cyanurate crystals

Project information:

Renal stone is a frequent disease with high recurrent rate because of absence effective cure method. Now, shock wave treatment is common and less painful technique to remove the stones. Actually, Chinese herbal medicine has been used to treat renal stones for many years. It is cheaper and has less side-effect. However, it is unable to be used extensively for the lack of scientific studies on it.

Participant only needs to take a Chinese herb power twice per day for 7 consecutive days. We shall closely monitor your health for any reaction. Large doses (>7 times of the given dosage) may cause anxiety and dizziness in sensitive individuals. Symptoms subside after stopping using the herb. Also, you require to leave early morning urine before, 1 day and 8 days after treatment for urine analysis.

Risk / Benefits:

The study hopes to prove that the studied Chinese herbal medicine has inhibitory effects on the formation of melamine cyanurate renal stones in vivo. If the result gives a positive effect, then efforts may be made to conduct a randomized control clinical trial. Lastly, Chinese herbal medicine can become another good (cheaper and less side effects) choice of treating renal stone or prophylactic agent use after shock wave treatment.

We hope that we can get some scientific evidences to support that traditional Chinese herbal medicine may be an alternative way to treat renal stones.

I _____ hereby consent to participate in the captioned research conducted by Ngork Wah Poon and his supervisor Dr. Mayur Danny Indulal GOHEL.

I understand that information obtained from this research may be used in future research and published. However, my right to privacy will be retained, i.e., my personal details will not be revealed.

The procedure as set out in the attached information sheet has been fully explained. I understand the benefits and risks involved. My participation in the project is voluntary.

I acknowledge that I have the right to question any part of the procedure and can withdraw at any time without penalty of any kind.

Name of participant: _____

Signature of participant: _____

Name of researcher: Ngork Wah Poon

Signature of researcher: _____

Date: _____

香 港 理 工 大 學

醫 療 科 技 及 資 訊 學 系

參 與 研 究 同 意 書

研 究 主 旨: 中 醫 中 藥 對 腎 石 症 的 功 效

研 究 內 容: 腎 石 症，是 一 種 常 見 和 復 發 率 高 的 腎 臟 疾 病，無 特 效 藥 物 可 以 治 療，體 外 碎 石 是 最 常 用 的 治 療 方 法，但 病 人 會 感 到 輕 微 痛 楚 及 不 適。其 實 經 多 年 累 積 臨 床 經 驗，中 醫 中 藥 排 石 治 療 有 一 定 的 成 效，一 方 面 費 用 低，且 無 副 作 用。但 中 醫 中 藥 缺 乏 科 學 研 究 證 據，因 此 一 直 未 廣 泛 運 用。

參 加 者 只 需 按 照 指 示，連 續 服 用 給 予 的 單 方 中 藥 七 天，而 且 在 療 程 前 和 完 成 療 程 後 留 下 小 便，用 作 研 究。研 究 人 員 將 會 密 切 留 意 參 加 者 服 藥 後 的 反 應

注 意：服 用 過 量 (超 過 7 倍 給 予 的 劑 量) 可 能 會 出 現 焦 慮 或 暈 眩，停 服 後，徵 狀 會 自 然 消 失。

我 們 希 望 通 過 今 次 科 研，取 得 數 據 ， 以 證 明 該 中 藥 有 壓 抑 腎 石 形 成 的 療 效。若 取 得 正 面 結 果 ， 大 型 的 臨 床 研 究 亦 可 隨 之 進 行。 最 後 令 該 中 藥 能 成 為 治 療 腎 石 症 的 更 佳 方 法 。

本 次 研 究 的 中 藥 與 現

本 人 _____ 同 意 參 加 由 Ngork Wah Poon 負 責 執 行, Dr. Mayur Danny Indulal GOHEL 監 督 的 研 究 項 目。

我 理 解 此 研 究 所 獲 得 的 資 料 可 用 於 未 來 的 研 究 和 學 術 交 流。然 而 我 有 權 保 護 自 己 的 隱 私，我 的 個 人 資 料 將 不 能 洩 漏。

我 對 所 附 資 料 的 有 關 步 驟 已 經 得 到 充 分 的 解 釋。我 理 解 可 能 會 出 現 的 風 險。我 是 自 願 參 與 這 項 研 究。我 理 解 我 有 權 在 研 究 過 程 中 提 出 問 題， 并 在 任 何 時 候 決 定 退 出 研 究 而 不 會 受 到 任 何 不 正 常 的 待 遇 或 責 任 追 究。

參 加 者 姓 名: _____

參 加 者 簽 名: _____

研 究 人 員 姓 名: Ngork Wah Poon

研 究 人 員 簽 字: _____

日 期: _____