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**Investigation of the effect of a solution of lime powder on urinary calcium oxalate kidney stone risk factors in artificial and real urines: *In vitro* and *in vivo* studies**

**Mpelegeng Victoria Bvumbi**



**Dissertation presented for the degree of Masters of Science  
In the Department of Chemistry  
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***Supervisor: Professor A. L. Rodgers***

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**“I know the meaning of plagiarism and declare that all of the work in the document, save for that which is properly acknowledged, is my own”.**

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*This dissertation is dedicated to the following people: my husband, Mr Elgin Bvumbi, my parents, Mr Selatole and Mrs Mammoge Teleki, my mother in law, Mrs Bvumbi and to my siblings, Ringo, Nan & Mmule. Thank you all for your encouragements, support and prayers.*

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## ***CONFERENCE PROCEEDINGS***

Mpelegeng Teleki, Allen Rodgers, Dawn Webber, Shameez Allie-Hamdulay. Investigation of the effect of a solution of lime powder on calcium oxalate crystallization in synthetic urine: preliminary results. *Urological Research* (2008) 36 (3-4): 195.

## ***CONFERENCE PRESENTATIONS***

- Poster presentation: 11<sup>th</sup> International Symposium on Urolithiasis. Nice, France, September 2008.
- Poster presentation: 1<sup>st</sup> Postgraduate Research Symposium. UCT, Cape Town, South Africa, October 2008.

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## **ABSTRACT**

**INTRODUCTION:** Dietary factors in the form of citrus juices (e.g orange, grapefruits, cranberry, lemonade and lime) appear to affect the ability of urine to inhibit calcium oxalate crystallization. In South Africa, previous studies have demonstrated that black and white individuals respond differently to lithogenic and antilithogenic dietary supplements.

**OBJECTIVES:** The current project was undertaken to study the inhibitory activity of lime in artificial urine and in the real urine of South African black and white subjects and also to assess the effects on urinary risk factors in these subjects after its ingestion.

**SUBJECTS AND METHODS:** Experiments commenced with the preparation of artificial urine (AU) in which the effects of lime solutions (0.125-1.00 mg/ml) were tested by carrying out various crystallization experiments. These included the determination of the calcium oxalate (CaOx) metastable limit (MSL); particle volume-size distribution (PSD), nucleation, aggregation and growth assays. Crystal deposition was studied using scanning electron microscopy (SEM).

*In vitro* experiments were then conducted in 24 hour urine samples from healthy South African black (n = 5) and white males (n = 5). The above-mentioned crystallization experiments were repeated in these urines. In addition, free Ca<sup>2+</sup> values and the Bonn risk index (BRI) of CaOx crystallization were determined.

Thereafter, a trial study in which 5 black and 4 white subjects ingested solutions of lime powder for 7 days was conducted. 24 hour urine samples were collected by subjects before and after the ingestion of lime. Urines were analysed for pH, sodium, potassium, calcium, oxalate, citrate, uric acid, chloride, magnesium, phosphate, and creatinine. Urine composition values were used as input data for the calculation of relative supersaturation (RS) values for calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD), brushite and uric acid using the computer programme EQUIL and Tiselius Risk Index (TRI) was also determined. The above-mentioned CaOx crystallization experiments were also performed. Urine compositions, crystallization data and physicochemical risk indices were analyzed statistically using analysis of variance (ANOVA).

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**RESULTS:** None of the lime solutions did affect the CaOx MSL of the AU or the real urine (*in vitro*). However CaOx crystal nucleation was promoted while CaOx crystal growth and aggregation were inhibited. In the trial study, after ingestion of lime solutions, significant increases in urinary magnesium (1.80 vs 2.75 mmol/24h,  $p = 0.0001$ ) and phosphate (20.9 vs 24.6 mmol/24h,  $p = 0.023$ ) were observed in black subjects. On the other hand, in white subjects, a significant decrease in urinary oxalate (0.313 vs 0.205 mmol/24h,  $p = 0.023$ ) and TRI (304 vs 187,  $p = 0.0102$ ) were noted. However, lime did not increase citrate levels to a significant degree in either group.

**CONCLUSIONS:** The results presented in this dissertation have shown that lime may be regarded as a potential therapeutic agent for reducing the risk of CaOx kidney stones in the white group based on the trial study. The results also provide further evidence in support of the hypothesis that there is a difference between the black and white populations with respect to their handling of lithogenic and antilithogenic dietary challenges. However, rigorous controlled trials in healthy subjects and in kidney stone patients need to be conducted in future studies to explicitly confirm these findings.

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## ***ABBREVIATIONS AND SYMBOLS***

ANOVA	Analysis of variance
AU	Artificial urine
A <sub>In</sub>	Aggregation inhibition
BMI	Body mass index
BU	Black urine
BRI	Bonn risk index
CaOx	Calcium oxalate
CaP	Calcium phosphate
COM	Calcium oxalate monohydrate
COD	Calcium oxalate dihydrate
COT	Calcium oxalate trihydrate
GAGs	Glycosaminoglycans
In	Inhibition
I <sub>G</sub>	Inhibition growth
MSL	Metastable limit
Na <sub>2</sub> Ox	Sodium oxalate
N <sub>In</sub>	Nucleation inhibition
OD	Optical density
P	Probabilities
PSD	Particle volume-size distribution
RE	Retinol equivalents
RS	Relative supersaturation
SE	Standard error
SEM	Scanning electron microscopy
THP	Tamm-Horsfall protein
UPTF1	Urinary prothrombin fragment-1
WU	White urine
Tris	Tris (hydroxymethyl)methylamine
XRD	x-ray powder diffraction

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**CHAPTER ONE**  
**GENERAL INTRODUCTION**

## **INTRODUCTION**

Urolithiasis is a medical term for kidney stone disease, the most common chronic kidney condition dating back to ancient times. It occurs throughout the world in about 8-20 % of the population in western societies (Pak 1998; Ramello et al. 2000; Daudon 2005; López and Hoppe 2008). Intriguingly, the incidence of these stones in the South African black population is rare compared to that of the South African white population, in which it is similar to that which occurs in other western societies (Muskat 1951; Modlin 1967; Whalley et al. 1998). This is of great interest as it provides basic scientists with a unique opportunity to study the characteristics that cause one group to be stone-free and another to be stone-prone. Numerous factors which could be biochemical, physicochemical and/or physiological in nature are likely to be involved in this phenomenon.

### **1.1 KIDNEY STONES AND THEIR COMPOSITION**

Urinary calculi are hardened mineral deposits that form in the kidneys, ureters or bladder. They form when the urine has various ion salts such as calcium and oxalate that nucleate to form calcium oxalate (CaOx) which build up in the urinary tract (Robertson et al. 1978 (a); Coe and Parks 1988; Kumar et al. 2003).

They start as crystals and develop into a stone over time. They may remain in the kidney or become lodged in the tube that carries urine from the kidney to the bladder called the ureter, and cause severe pain that begins in the lower back. The lodged stone can block the flow of urine, causing pressure to build in the affected ureter and kidney. Small stones can spontaneously pass out of the body on their own, even though this can be painful, whilst large stones may require surgery or shattering into smaller pieces with sound waves in a procedure called extracorporeal shockwave lithotripsy.

There are several types of urinary stones based on chemical composition, which depend on the chemical imbalance in the urine at the time of nucleation and growth. These can be divided into 4 main stone types namely calcium (approximately 80 %), uric acid (9 %), magnesium ammonium phosphate (“struvite”) (10 %) and cystine (1 %) (Finlayson 1978; Coe and Parks 1988; Morton et al. 2002; Coe et al. 2005; Moe 2006).

### **CaOx stones**

The most common cause of calcium stone production is excess calcium in the urine, clinically known as hypercalciuria. CaOx urolithiasis is a very common disorder and these stones may be pure or mixed, usually with calcium phosphate, uric acid or ammonium urate (Trinchieri et al. 2005). Calcium oxalate monohydrate (COM) (whewellite) and calcium oxalate dihydrate (COD) (weddelite) occur commonly, but calcium oxalate trihydrate (COT) is extremely rare (Herring 1962; Streit et al. 1998; Grases et al. 1998; Bithelis et al. 2004; El-Shall et al. 2004). The pathogenesis of idiopathic CaOx stones formation is multifactorial and these include epidemiological, metabolic, physicochemical and dietary factors. All of these will be discussed in the following pages. Because of the predominance of these stones; they constitute the focus of this dissertation.

### **CaP stones**

Calcium phosphate (CaP) stones typically occur in patients with metabolic or hormonal disorders such as hyperparathyroidism and renal tubular acidosis (Hess and Jaeger 1993). CaP stones exist in numerous forms and amongst them, apatite (calcium hydroxyl phosphate) and brushite (calcium hydrogen phosphate) are seen more frequently in women (Parks et al. 2004; Worcester and Coe 2008). Brushite stones are very physically resistant structures which are difficult to break with shock waves and therefore surgery is required to remove this type of stone (Klee et al. 1991). On the other hand, apatite stones are easily broken by shock wave (Williams Jr et al. 2003; Parks et al. 2004; Evan et al. 2005). The most important factors for the formation of CaP stones are high urinary pH (except for brushite stones which form at a pH below 6.5, Parks et al. 2004), high urinary calcium and low urinary citrate (Pak et al. 2008 (a)).

**Uric acid stones**

Uric acid is the end product of purine degradation in humans (Coe et al. 2005). It is a weak acid with pKa of 5.35 in urine. Approximately 3 % of stone-formers form uric acid stones (Nordin and Hodgkinson 1967). These stones are the consequence of abnormalities in purine metabolism, urate/uric acid renal handling and/or excess dietary protein (William-Larson 1990). Hyperuricosuria, low urinary volume and pH values below 5.5 are the well known contributing factors of uric acid stone formation (Shekarriz and Stoller 2002). The treatment of uric acid stones includes increased intake of fluid, urine alkalization of pH to values between 6.2 and 6.8 by ingestion of potassium citrate or sodium bicarbonate and adoption of a low-purine diet (Ferrari and Bonny 2004).

**Magnesium ammonium phosphate stones**

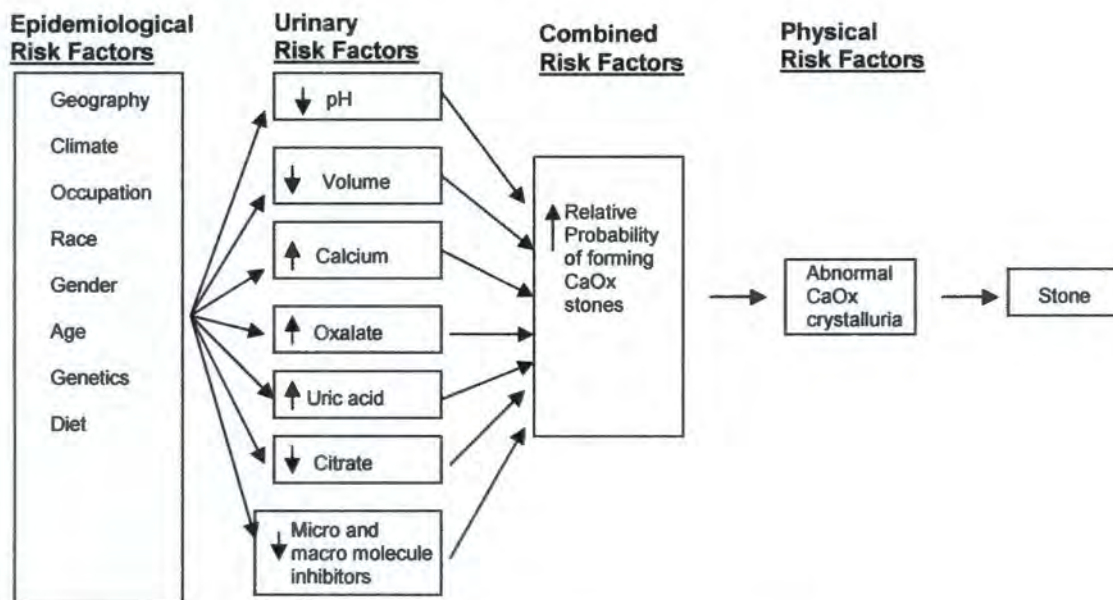
Magnesium ammonium phosphates (struvite) grow rapidly forming “staghorn-calculi”. The primary cause of these stones is chronic urinary tract infection (Bruyeni et al. 2008; Gómez-Núñez et al. 2009). This occurs as a result of micro-organisms such as *Proteus*, *Providencia*, *Klebsiella*, *Pseudomonas* and *Enterococci* which are hydrolysed by the enzyme urease which degrade urea to ammonia and CO<sub>2</sub>, raising the urine pH. These conditions favour the formation of struvite (Ramello et al. 2000).

**Cystine stones**

Cystine kidney stones occur due to cystinuria, an inherited disorder of the transport of an amino acid called cystine. These stones occur more commonly in children and young adults than in adults (Font-Llitjós et al. 2005). Cystine stones represent only a small percentage (1-2 %) of urinary calculi (Ramello et al. 2000; Coe et al. 2005).

## 1.2 AETIOLOGY

Aetiological factors for calcium oxalate urolithiasis can be divided into four main categories – epidemiological, urinary, combined and physical as shown in Figure 1.1. These factors will be discussed briefly in this chapter (Robertson and Peacock 1983).



**Figure 1.1** A risk factor model for calcium oxalate stone-formation in the urinary tract (*modified from Robertson and Peacock 1983*)

### Epidemiological Factors

#### 1.2.1 Geography and climate

The probability of forming stones varies in different parts of the world. For example in Saudi Arabia, the prevalence of kidney stones formation is about 20% (Robertson and Hughes 1994). In other developed countries, the risk of forming stones is reported as 13 % in USA, 12 % in Canada, 5-9 % in Europe and 1-5 % in Asia (Ramello et al. 2000). People who live in hot or warm climates or those who are exposed to very hot conditions are susceptible to kidney stone formation. It is likely that this is caused by dehydration with a resultant reduction of urine flow rate and a concomitant increase in the concentration of solutes in the urine (Blacklock 1982).

Notwithstanding this, the hypothesis that climate has an effect on the risk of kidney stone formation is not entirely consistent. This is demonstrated by the observation that in some regions of Africa, e.g Nigeria (Esho 1978; Olapade-Olaope et al. 2004), Southern Sudan (Kambal et al. 1981), Tanzania (Mkony et al. 1991), and South Africa (amongst the black population) (Wise and Kark 1961; Modlin 1967; Whalley et al. 1998), the occurrence of stones is rare.

### **1.2.2 Occupation**

Many studies have found that there is a higher incidence of stone formation amongst certain occupation groups, for instance people in sedentary occupations such as aviation pilots and truck drivers (Blacklock 1982; Ferrie and Scott 1984; Borghi et al. 1993; Zheng et al. 2002). A possible explanation could be that irregular and insufficient intake of fluids leads to increase urinary concentration of stone forming salts.

### **1.2.3 Race, Gender and Age**

Generally the incidence of kidney stones has been reported to be relatively higher in whites compared to blacks, irrespective of geographical area (Whalley et al. 1998; Ramello et al. 2000; Taylor and Curhan 2007). For example, reports have shown that urolithiasis in the USA is more prevalent in whites than in blacks (Finlayson and Reid 1978; Soucie et al. 1994), and these reports have been recently supported by Maloney et al. (2005) who showed that of 1,141 stone patients from the same region (North Carolina, USA) who participated in a metabolic evaluation study, only 9 % were non-white. Other groups have also been reported to show stone rarity, namely Indians in Mexico, Peru, Ecuador and Bolivia (Finlayson 1978), Inuit and Australia's aboriginal population (Scott and Mowart 1977).

In South Africa, about 15 % of the white population are at risk of developing kidney stone disease in their life time. On the other hand less than 1 % of the black population form stones (Modlin 1967; Meyers et al. 1994; Whalley et al.1998). The latter phenomenon forms the primary motivation for undertaking the research described in this thesis.

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Many studies have shown that urolithiasis is age-dependent, with about 15 % of men and 5 % of women being afflicted by the time they are 30-60 years old (Ljunghall et al. 1977; Hesse et al. 1986; Blacklock 1982; Yoshida and Okada 1990; Iguchi et al. 1999). It is not clear why women have a lower incidence of stones, but a few possible explanations have been proposed. The first is that elevated uric acid excretion rates occur in a greater proportion in males than in females (Coe FL 1978). Second, there is evidence that oestrogen protects women against kidney stone (Liao and Richardson 1972; Finlayson 1974; Heller et al. 2002). Another possible explanation might be that female subjects have a higher level of urinary citrate that might protect them from calcium urolithiasis (Welshman and McGeown 1975). Conversely, testosterone may increase the risk of stone formation by increasing urinary oxalate excretion in males (Lee et al. 1996).

#### **1.2.4. Genetics**

Urolithiasis is also a genetically-related disease. In a study by Ljunghall et al. (1985), 55 % of patients disclosed that they had at least one first-degree relative with renal stone history. In Italy; Trinchieri et al. (1988) observed that 37 % of cases and Gambaro et al. (1996) reported that 38 % of non-current and 50 % of current stone formers have a positive family history of nephrolithiasis. In the USA, Curhan et al. (1997 (a)) in a study of 37 999 men investigated an interaction between family history and dietary factors and found that males who had kidney stones are approximately 3 times more likely to have a family history of this disease than females. In another study, 36 % of 1223 women reported a family history of stones (Curhan et al. 2004).

#### **1.2.5 Diet**

Many research studies have demonstrated the relationship between dietary intake of various nutrients and the excretion of lithogenic and inhibitory substances in the urine (Hesse et al. 1993; Curhan 1993, 1996, 1997 (b), 2004; Massey et al. 1993, 2003; Rodgers 1997, 1998, Siener and Hesse 2002; Siener et al. 2004; Taylor and Curhan 2004; Karagülle et al. 2007).

**Dietary oxalate**

Urinary oxalate is derived from endogenous and exogenous sources. Endogenous oxalate production is directly proportional to body weight (Massey 2003). It is synthesised from two major sources: approximately 40 % is derived from the end product of ascorbate metabolism and approximately 40-50 % is derived from the metabolic reaction involving glyoxalic acid (Massey et al. 1993; Robertson 1999). Exogenous oxalate is derived from dietary sources occurring in various plant foods (Massey 2003). Approximately half of ingested oxalate is used as a substrate by enteric bacteria, and about 25 % is excreted unchanged in the faeces (Robertson 1999). Dietary oxalate influences the level of urinary oxalate (Kasidas and Rose 1980; Hesse et al. 1993; Holmes and Kennedy 2000; Holmes et al. 2001). As a consequence, CaOx stone patients should be advised to reduce their intake of oxalate-rich foods. Spinach, rhubarb, beetroot, nuts, chocolate, legumes, parsley, strawberries, concentrated brans and tea contain high levels of oxalate which can cause a significant increase in urinary oxalate excretion (Finch et al. 1981; Brinkley et al. 1990; Hesse et al. 1993; Massey et al. 1993, 2003; Holmes et al. 2001), and therefore promote the formation of CaOx stones.

Interestingly, co-ingestion with foods rich in calcium (e.g. dairy products) minimizes the rise in urinary oxalate by binding oxalate in the intestinal tract (Curhan et al. 1993; Curhan et al. 2004). On the other hand, the rise in urinary oxalate from dietary oxalate is more marked when dietary calcium is restricted. (Curhan et al. 1993, 1997 (b), 2004; Krieg 2005).

**Dietary calcium**

A high calcium intake is a potential risk factor for stone formation as it raises urinary calcium and the saturation of calcium salts (Barilla et al. 1978; Bataille et al. 1983). However, a high calcium diet may also lower urinary oxalate by binding oxalate in the intestinal tract (Curhan et al. 1993, 1997 (b); Hess et al. 1998; Krieg 2005).

This will oppose the rise in urinary oxalate and thereby may prevent the formation of CaOx crystals in urine (Curhan et al. 1997 (b)). There have been contradicting results with regard to whether a high or low calcium intake is suitable for CaOx stone patients. For many years, patients were advised to reduce their dietary intake of calcium (Bleich et al. 1979; Robertson et al. 1981; Robertson 1987).

But more recent studies have demonstrated that this is inappropriate and even dangerous as the reduction of calcium intake might not only increase the risk of CaOx stone formation (by inducing higher absorption and hence higher urinary excretion of oxalate) but could also causes a negative calcium balance and further loss of bone tissue (Curhan 1993; Messa et al. 1997; Hess et al. 1998; Curhan 1999; Lemann 2002).

Interestingly, Heller et al. (2003) recently reported that a high calcium diet conferred an alkali load that produced an increase in urinary pH, potassium, phosphorus and citrate but significantly increased relative saturation ratio of CaOx by 24 %.

It is important to mention that the pathogenic role of dietary calcium on stone formation depends on the state of intestinal calcium absorption and on the degree of dietary oxalate restriction. Thus, dietary advice for CaOx patients should be focused on maintaining an intake between 800 and 1200 mg of calcium per day rather than restricting or exceeding the patient's normal intake (Wahl and Hess 2000, Taylor and Curhan 2004).

#### **Dietary animal protein**

An increase in animal protein consumption has been reported to cause an increase in urinary calcium and uric acid excretion (Roberson et al. 1979 (b); Kerstetter 2003; Pak 2004).

Studies have demonstrated a relationship between the incidence of stone disease and consumption of animal protein (Robertson et al. 1979 (a) and (b); Brockis et al. 1982; Hesse et al. 1993; Curhan et al. 1993). In a study by Brockis et al. (1982), dietary habits and urine composition of 30 meat-eating subjects were compared with those of vegetarian subjects. They found that urinary calcium excretion increased as animal protein intake increased. Curhan et al. (1993) showed in a prospective study of dietary calcium and other nutrients a 33 % increase in risk of stone disease in men who had no history of stone disease who were taking  $\geq 77$  g/day of animal protein as compared to those who were taking  $\leq 50$  g/day of animal protein. The proposed mechanism is that high animal protein load reduces urinary pH and citrate and increases calcium (Reddy et al. 2002; Siener and Hesse 2002). Moreover, it increases urinary uric acid due to its high content of purine which is a substrate for urate synthesis (Coe et al. 1976; Krieg 2005).

Advice given to kidney stone formers is that they should consume between 0.8 and 1.2 g of meat protein per kg body weight per day (Wahl and Hess 2000; Kerstetter et al. 2003).

#### **Other dietary factors**

Several studies have reported that high carbohydrate intake results in increased urinary calcium excretion (Raskin et al. 1978; Wood and Allen 1983; Iguchi et al. 1993; Coe and Parks 1994; Taylor and Curhan 2004). Carbohydrates also stimulate endogenous synthesis of oxalate which is eventually excreted in urine (Lekcharoensuk et al. 2001). With high urinary concentrations of calcium and oxalate, the risk of forming stones is more pronounced as these are the main urinary CaOx risk factors. Therefore a low carbohydrate diet should be recommended to stone-forming patients.

The effect of dietary sodium on CaOx stone formation has been studied mainly because of its calciuric action. Researchers are in agreement that for every increase in dietary NaCl of 100 mmol (i.e 2300 mg Na), there is an approximate increase of 1.0 mmol (40 mg) in urinary calcium excretion (Lemann et al. 1995; Blackwood et al. 1999). Studies by Kok et al. (1990) and Shakaee et al. (1993) found that increasing dietary sodium chloride (from 14 to 310 mmol/day and 50 to 250 mmol/day respectively) increased urinary calcium by 34 % and 31 % respectively and decreased urinary citrate by 10 % and 20 % respectively. Thus, hypercalciuria and hypocitraturia are important lithogenic consequences of excessive intake of dietary salt, thereby increasing the risk of CaOx stone formation.

Therefore CaOx stone formers are advised to decrease their sodium intake to less than 6g of dietary salt per day. Most patients can accomplish this by avoiding salty foods and salt shakers (Pak 2004).

#### **Fluid intake**

Increased fluid intake is of great importance in preventing urolithiasis (Pak et al. 1980; Borghi et al. 1996). This is because a low fluid intake leads to concentrated urine and increases the risk of stone formation. Thus, it is common practice to advise all stone forming patients, regardless of their stone type to consume between 2.5 and 3 litre of fluid per day to maintain a urine output of 2 litres a day (Pak 1984; Goldfarb 1988; Goldfarb 1990).

A urine volume of less than 1 litre a day is the only scientifically proven factor known to increase the risk of CaOx stone formation (Goldfarb 1990).

Water is the best fluid to drink in order to avoid the risk of kidney stones (Krieg 2005) and mineral water is believed to be an ideal beverage to dilute the urine (Hesse et al. 1993). Numerous studies have been undertaken on mineral water (rich in calcium, magnesium and bicarbonate) on either healthy subjects or patients with kidney stones by different researchers (Hesse et al. 1993, Rodgers 1997, 1998; Trinchieri et al. 1999; Siener et al. 2004; Karagülle et al. 2007). Water which is rich in magnesium and bicarbonate was found to increase inhibitory factors such as citrate and magnesium and to increase urinary pH as well. The same impact was observed for South African and French mineral waters which are rich in magnesium and calcium (Rodgers 1997, 1998).

Even though the term 'fluid intake' in most cases implies water intake, other fluids like citrus juices are also recommended. These will be discussed in details in section 1.6 as they form a major part of this MSc dissertation.

### **1.3 STATES OF SUPERSATURATION**

The physicochemical theory of stone formation involves supersaturated solutions and the effects of inhibitors and promoters of the fundamental mechanisms of stone formation, namely nucleation, growth and aggregation (Finlayson 1978; Rose 1982; Kok and Khan 1994; Hess and Kok 1996, Coe et al. 2005).

#### **1.3.1 Fundamental role of supesaturation**

Supersaturation is a prerequisite for the occurrence of crystallization in urine (Finlayson 1978). It is the thermodynamic driving force for the change of phase from solution to solid (crystallization) and it is a representation of excess free energy between the two phases (Hess and Kok 1996; Kavanagh 2006 (a)). As such, it is an important condition for renal precipitation and stone formation. This driving force or free energy ( $\Delta G$ ) is given by the equation

$$\Delta G = RT \ln (A/A_{eq})$$

where R is the gas constant, T is the temperature, A is the activity of the unionised salt in the supersaturate solution and  $A_{eq}$  is the activity of the solution at equilibrium (Finlayson

1978; Hess and Kok 1996; Kavanagh 2006 (a), (b)). ( $A/A_{eq}$ ) is also referred to as relative supersaturation (RS). Therefore, when  $RS < 1$ , the solution is said to be undersaturated and any stone forming salts that are present can freely dissolve. But when  $RS = 1$ , the solution is saturated, and when  $RS > 1$ , the solution is supersaturated.

### **1.3.2 Thermodynamics of crystallization**

Crystallization is the general term for the formation of crystalline material. The driving force for crystallization is the presence of supersaturation with respect to the composing salts. This is therefore referred to as the thermodynamic driving force of crystallization (Hess and Kok 1996). When this condition is met, the following kinetic mechanisms (nucleation, growth and aggregation) occur. These processes are all discussed below.

#### **Nucleation**

Nucleation is known as the initial phase transformation that leads to the formation of urinary stones (Finlayson 1978; Finlayson et al. 1984). This process occurs in a supersaturated solution. It starts with the clustering of stone salts in solution into loose conglomerates which can grow due to the decrease in free energy that is associated with change from liquid to solid phase (Finlayson 1978; Finlayson et al. 1984; Hess and Kok 1996).

Urine is rarely sufficiently concentrated to ensure a reasonable rate of spontaneous or homogeneous nucleation. Thus, it is probable that nucleation usually takes place on a foreign surface, i.e heterogeneous nucleation (Finlayson 1978; Finlayson et al. 1984; Hess and Kok 1996).

#### **Growth**

Once a crystal nucleus has reached its critical size, new crystal components are added to reduce the overall free energy. This mechanism is known as crystal growth. Crystal growth occurs due to 3 processes called speciation, bulk diffusion and adsorption.

Speciation refers to the binding of monomers to form complexes and these monomers migrate through the solution by a process called bulk diffusion. Bulk diffusion refers to the process in which the newly formed crystals travel through the solution and attach to the crystal by adsorption (Hess and Kok 1996).

### **Aggregation**

Aggregation refers to the process whereby crystals combine to form a large particle under all states of saturation. There are two different types of aggregation, namely primary and secondary aggregation (Söhnel and Grases 1995). The primary aggregation represents a crystal malgrowth resulting in the formation of a polycrystalline particle while secondary aggregation proceeds by collision of particles suspended in the liquid induced by shear forces acting in the liquid.

Crystal aggregation compared to nucleation and growth, is more important in the process of stone formation because CaOx growth is too slow to produce clinically significant particles within the transit time in the urinary tract, while aggregation occurs within seconds and is thus more dangerous for the formation of large crystalline particles in renal tubules (Kok et al. 1988; Hess and Kok 1996).

## **1.4 URINARY RISK FACTORS**

Urolithiasis has long been recognised as the combined result of a number of individual determinant factors which are called biochemical risk factors (Robertson et al. 1978; Finlayson et al. 1984; Hess and Kok. 1996). These risk factors can be considered as those determining the degree of supersaturation of urine, and affect the tendency of the crystals to enlarge and aggregate once nucleation and growth has occurred. The urinary risk factors which are measured in 24 hour urine samples are pH, volume, calcium, oxalate, uric acid, magnesium and citrate. Each of these risk factors is discussed below.

### **1.4.1 Urinary pH**

Urinary pH is one of the most important risk factors of stone formation because it can influence inhibition and promotion of stone formation.

An alkaline urinary pH reduces the risk of CaOx crystallization (Bilobrov et al. 1990; Pak 1994). This is because phosphate and citrate ions dissociate at high pH, thereby increasing the complexation of calcium which lowers the urinary saturation of CaOx (Pak 1994). But if hypercalciuria is present, then the increased complexation between calcium and phosphate that occurs with increasing alkalinity of the urine may increase the risk of CaP crystallization (Tiselius 1984) because CaP stone formation is most likely to occur at  $\text{pH} > 6$  (Bilobrov et al. 1990).

Thus, Tiselius (1981) has suggested that patients with hypercalciuria who are on therapeutic alkalinization, the therapy should be combined with calcium reducing measures to avoid brushite stone formation.

On the other hand, a pH less than 5.5 may promote uric acid stone formation because of the predominance of the undissociated uric acid (Bilobrov et al. 1990; Tiselius 2003).

It is therefore advisable to maintain the urinary pH between 6 and 7 because this range exhibits high inhibition of CaOx crystallization and uric acid formation (Bilobrov et al. 1990).

#### **1.4.2 Urine volume**

Urine volume is also extremely important in the pathogenesis of urolithiasis. A high urinary volume reduces the relative supersaturation of the crystal components in urine as a consequence of dilution effects. In addition, a high urinary volume leads to high urine flow rates, which will flush out the crystals that have formed in the urinary tract. As a result, high fluid intake has been the recommended treatment for kidney stones (Pak et al. 1980; Borghi et al. 1996; Borghi et al. 1999). In a study by Pak et al. (1980), in which they investigated the effect of diluting urine, they found that a high water intake could inhibit the formation of kidney stones by reducing the saturation of calcium salts.

A randomized clinical trial which lasted 5 years by Borghi et al. (1996) also demonstrated the value of increasing urine volume. In that study, stones recurred in only 12 of the 99 patients who maintained a urine volume of about 2.6 L/day over 5 years, while stones recurred in 27 of the 100 patients in the control group whose urine volume was about 1.2 L/day.

As mentioned before in section 1.2, fluids other than water can be consumed to achieve a higher urinary volume. These include among others, citrus juices which will be discussed in detail later in this thesis, and herbal teas (Hesse et al. 1993). Because of an increase in the supersaturation of stone forming salts due to low volume of urine, stone forming patients should be advised to drink more fluids in order to achieve a urine output of 2 or more litres a day. However, other fluids that must be avoided are those with high oxalate content, for example cola (Weiss et al. 1992; Rodgers 1999 (a)) and hot chocolate and large amounts of tea (Hesse et al. 1993). Other fluids which must be avoided as they increased the risk of kidney stones include apple juice and grapefruit juice (Curhan et al. 1996).

### **1.4.3 Urinary calcium**

Hypercalciuria occurs to a greater extent in stone formers as compared to normal controls (Heilberg 2000; Curhan et al. 2001) and is induced in about half of recurrent kidney stone formers (Morton et al. 2002). It is a risk factor for recurrent stone formation (Coe et al. 1982; Vahlensieck 1986; Goldfarb 1994; Borghi et al. 1996). A normal upper limit for 24h urinary calcium excretion of 7.5 mmol and 6.5 mmol in men and women respectively has been reported (Monk 1996; Bihl and Meyers 2001). For many years, hypercalciuria was regarded as one of the most important factors that contribute to a high supersaturation of CaOx urolithiasis (Marshall et al. 1972; Bleich 1979; Vahlensieck 1986; Rose 1987). However, as described earlier, it is urinary oxalate which is more important in the formation of CaOx crystals than calcium (Borsatti 1991). Because hypercalciuria is common, a moderate calcium intake of 1 g/day may be a reasonable recommendation for CaOx patients (Morton et al. 2002).

### **1.4.4. Urinary oxalate**

Oxalate is a dicarboxylate anion which forms an insoluble salt with calcium in urine. It is the end product of several metabolic pathways, including those involving serine, glycine, hydroxyproline and ascorbate. (Morton et al 2002). It is by far the most critical determinant of stone prevalence compared to other risk factors like hypercalciuria, hyperuricosuria and hypocitraturia (Ito et al. 1989; Borsatti 1991; Ito et al. 1992).

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There is much less oxalate than calcium in urine; therefore small increases in oxalate concentration have a far greater impact than changes in calcium concentrations. A study by Rodgers (1999 (b)) has demonstrated this by showing that the rate of change in supersaturation is 23 times greater for increased oxalate than for comparable increases in calcium.

Various mechanisms have been identified for increases in urinary oxalate excretion. Firstly, an increased dietary intake allows more oxalate to reach the colon, increasing its availability for absorption (Robertson 1999). A second mechanism is an increased endogenous production of oxalate (Massey et al. 1993). Finally a deficiency in oxalate-degrading bacteria (Allison et al. 1986, Sidhu et al. 1999) particularly *Oxalobacter formigenes* has been suggested as causing an increase in the risk of hyperoxaluria.

#### **1.4.5. Urinary uric acid**

High urinary excretion of uric acid (hyperuricosuria) is a risk factor for uric acid and CaOx stone formation. Two possible mechanisms have been suggested for the elevation of urinary uric acid: excessive intake of dietary protein and endogenous overproduction of uric acid (Coe 1978). It is most commonly caused by increased purine intake in the diet (Halabe and Sperling 1994; Porena et al. 2007). On the basis of the observation that approximately one third of CaOx stone formers have elevated urinary uric acid excretion (Coe 1978), elevated excretion of urinary uric acid has been proposed as a risk factor for the nucleation, growth and aggregation of CaOx crystals (Robertson et al. 1978 (b); Griffith et al. 1986).

Treatment with allopurinol blocks uric acid production and decreases the recurrent rate of CaOx kidney stones in patients with hyperuricosuria (Pak 1978; Ettinger 1991), even though this treatment has no effect on urinary calcium or oxalate saturation (Baggio et al. 1983; Tiselius et al. 1986).

#### **1.4.6 Urinary magnesium**

Several decades ago it was established that magnesium can decrease the crystallization of CaOx by complexing with oxalate ions to form soluble magnesium oxalate, thus reducing

the amount of free oxalate in the urine (Desmars and Tawashi 1973; Hallson et al. 1982). It is this capacity of magnesium to chelate oxalate that makes it an inhibitor of CaOx crystallization. This is advantageous since, as mentioned earlier, oxalate has a far greater impact in CaOx stone formation than calcium.

#### **1.4.7 Urinary citrate**

Hypocitraturia is a major risk factor for kidney stones (Morton et al. 2002; Porena 2007). Several studies have demonstrated hypocitraturia in stone formers (19-63 %), thereby showing its importance in the pathogenesis of this disease (Rudman et al. 1982; Nicar et al. 1983; Pak et al. 1985; Laminski et al. 1990; Cupisti et al. 1992).

It is caused by high animal protein diet which leads to reduction of urinary citrate (Pak 2004). Other causes for hypocitraturia are distal renal tubular acidosis, and metabolic acidosis of chronic diarrheal syndrome (Pak 2004). Hypocitraturia increases urinary saturation of CaOx by impairing the formation of calcium citrate complex  $[CaCit]^{-1}$ . It also promotes nucleation and aggregation of CaOx and crystal growth of CaP (Pak 2008 (b)).

Hypocitraturia can be treated, and stone patients with this disorder are recommended to undergo alkali therapy (Parks and Coe 1986; Conte et al. 1989 (a)) which includes the intake of K-citrate or Na-K-citrate supplements (Pak et al. 1983, 1985; Porena et al 2007). Not only can therapy be used to treat hypocitraturia, but also citrus fruits/juices have been found to be of importance with respect to treatment of stones. As mentioned before, this topic will be discussed fully in section 1.6 of this Chapter.

### **1.5 URINARY INHIBITORS**

An inhibitor is any substance, molecule, ion or agent that slows down or prevents nucleation, growth or aggregation of calcium salts in urine (Kok 1996; Worcester 1996; Ryall 1997). They may act by complexing either calcium or oxalate ions, thereby reducing the level of CaOx supersaturation in the crystallizing solution.

Urinary inhibitors can be categorised into micromolecules which include: multivalent metallic cations (e.g magnesium), small organic (e.g citrate) or inorganic (e.g pyrophosphate) anions and macromolecules such as glycosaminoglycans and proteins (Worcester 1996; Moe 2006).

### **1.5.1 MICRO-INHIBITORS**

**Magnesium:** Magnesium has been shown to decrease the crystallization of CaOx by complexing with oxalate ions to form soluble magnesium oxalate. This means that magnesium can reduce the amount of free oxalate ions in the urine (Desmars and Tawashi 1973; Hallson et al. 1982; Danielson 1985). This is favourable because as mentioned earlier, oxalate is the determining factor in CaOx formation. Studies have shown that magnesium lowers the urinary saturation, decreases nucleation and growth of CaOx and even CaP crystals (Kohri et al. 1988; Grases et al. 1989 (a); Lindberg et al. 1990; Pak et al. 1992).

**Pyrophosphate:** Pyrophosphate inhibits nucleation of CaOx crystals (Grases et al. 1989 (b); Ryall 1997), causes some inhibitory effects of crystal growth (Grases et al. 1989 (c); Conte et al. 1989 (b)) and is a strong inhibitor of aggregation of CaOx crystals (Robertson et al. 1973; Robertson et al. 1974; Felix et al. 1977). It inhibits these processes by binding calcium in its solid phase. It irreversibly binds to COM crystal surfaces and not to COD (Shirane and Kagawa 1993; Wesson et al. 1998). This is of great pathological importance since COD crystals are less adherent to renal epithelial cells than COM crystals, thus potentially inhibiting a critical step in the genesis of kidney stones (Shirane and Kagawa 1993; Wesson et al. 1998).

**Citrate:** Citrate is an important urinary acid neutralizer and it is a naturally occurring urinary stone inhibitor that chelates calcium in solution, forming a highly soluble calcium-citrate complex that decreases ionic concentration of calcium, thereby decreasing the RS of CaOx and CaP in urine (Preminger et al. 1985; Pak et al. 1985).

It also inhibits their crystal nucleation, growth (Ryall et al. 1981; Pak 1987; Grases et al. 1989 (c); Grases and Costa-Bauza 1990; Conte et al. 1990; Laube et al. 2002) and aggregation (Kok et al. 1987; Tiselius et al. 1993 (a) and (b)).

Citrate is derived from both endogenous (tricarboxylic cycle) and exogenous sources (citrus fruits) (Morton et al. 2002).

### **Supplemental citrate**

Supplemental citrate is the most common therapeutic agent that is used to prevent calcium and uric acid stones. The inhibitory potency of citrate depends on its ability to alkalize urine and to form complexes with calcium. Such complexation reduces urinary supersaturation for CaOx and CaP and retards the nucleation, growth and aggregation as well as agglomeration of preformed CaOx crystals (Pak and Fuller 1983; Kok et al. 1986; Laube et al. 2002; Byer and Khan 2005).

The therapeutic effects of various different citrates on CaOx crystallization inhibition have been tested. Potassium citrate is the most common supplementation as it has been demonstrated to increase urinary citrate levels, and decrease urinary calcium excretion and relative supersaturation with respect to CaOx (Sakhaee et al. 1983; Pak et al. 1985; Preminger 1988; Hofbauer et al. 1994; Whalley et al. 1996; Sellmeyer et al. 2002).

Other citrate salts that have been tested include sodium citrate (Sakhaee et al. 1983, Allie-Hamdulay and Rodgers 2005), magnesium citrate (Schwille et al. 1999), potassium-magnesium citrate (Pak et al. 1992; Ettinger et al. 1997), calcium citrate (Sakhaee 2005), calcium-sodium citrate (Schwille et al. 1997) and sodium-potassium citrate (Achilles et al. 1990; Hofbauer et al. 1994).

Some of these therapies have shown 60 to 96 % reduction in kidney stone recurrence (Pak and Fuller 1983; Pak et al. 1985, Pak and Peterson 1986; Ettinger et al. 1997).

However, even though the alkali citrate therapy showed some reductions on CaOx stones, it can be a burden as some patients experience gastric side effects and financial constraints (Schwille et al. 1992; Hofbauer et al. 1994; Seltzer et al. 1996).

### 1.5.2 MACROMOLECULES

In the context of urolithiasis, 'macromolecules' refer to urinary glycosaminoglycans and proteins. Numerous studies have shown that normal urine contains macromolecular substances that are capable of preventing and reducing CaOx crystal formation and aggregation (Boyce 1968; Morse and Resnick 1989; Jones and Resnick 1990; Ryall 2000; Khan and Kok 2004).

#### Glycosaminoglycans (GAGs)

GAGs are polysaccharides which are composed of repeating disaccharide units (Roberts and Resnick 1986). They have a varying molecular weight of 18-40 kDa and account for 20 % of the matrix of stone. Although there are *in vitro* studies which have demonstrated that they inhibit growth and aggregation of CaOx crystals (Roberts and Resnick; Ryall 1997; Khan and Kok 2004), some studies have demonstrated that GAGs promote nucleation and growth rate (Kohri et al. 1989).

#### Urinary proteins

The organic matrix of kidney stones comprises 2.5 % of dry weight containing lipids, carbohydrates and proteins. The latter comprise 64 % of the stone matrix itself (Boyce and Garvey 1956). Albumin,  $\alpha$  and  $\gamma$ -globulin and Tamm-Horsfall Protein (THP) were the first proteins to be identified in the stone matrix (Boyce et al. 1968), while other proteins including nephrocalcin, osteopontin, albumin, urinary prothrombin fragment 1 (UPTF1), inter- $\alpha$ - trypsin inhibitor and bikunin have been identified to be having a role in the inhibition and promotion of CaOx crystallization (Rose et al. 1982; Nakagawa et al. 1983; Coe et al. 1991; Doyle et al. 1991; Ryall et al. 1991; Atmani et al. 1993; Hess et al. 1993; Worcester and Beshensky 1995; Grover et al. 1998; Fries and Blom 2000; Webber et al. 2002; Yang et al. 2005).

**Nephrocalcin:** Nephrocalcin is a glycoprotein with a molecular weight of ~ 14 kDa. It has been reported to self-aggregate into large molecules that range from 23-68 kDa

(Worcester et al. 1987). Nephrocalcin has been demonstrated to inhibit the crystal formation and aggregation as well as being able to prevent attachment of crystals on the surface of renal epithelial cells (Nakagawa et al. 1983).

**Osteopontin:** Osteopontin was originally isolated as a glycoprotein with molecular weight of 50 kDa (Khan and Kok 2004). It is found in urine, human CaOx stones, CaOx and CaP crystals grown in human urine (Worcester 1996; Wesson et al. 1998). Osteopontin can modulate CaOx crystallization by inhibiting crystal nucleation, growth and aggregation (Worcester 1996; Wesson et al. 1998).

**Tamm-Horsfall Protein (THP):** THP is the most abundant protein in human urine with molecular weight of 80-100kDa (Ryall 1997). It is produced in the kidney and specially localized in the epithelial cells of the loop of Henle. Some studies report that THP inhibits and promotes CaOx crystallization depending on the experimental conditions (Hess 1994; Grover et al. 1998) while others have reported that THP can inhibits nucleation and aggregation of CaOx and CaP crystallization (Rose et al. 1982).

**Albumin:** Albumin has also been classified as the most abundant protein in the urine (Hess et al. 1989; Grover et al. 1998). It is manufactured in the liver and has been detected as the major component in the matrix of all urinary stones (Boyce 1968). It has been shown to promote CaOx nucleation but inhibits CaOx crystal aggregation (Grover et al. 1998; Worcester 1996; Khan and Kok 2004).

**Prothrombin Fragment 1 (UPTF1):** UPTF1 is a protein with a molecular weight of 31 kDa. This protein is found in calcium stones and also located in greater quantities in the human kidney of the stone formers (Doyle et al. 1991, 1995). It was found to be an effective inhibitor of CaOx growth and aggregation in ultrafiltered urine (Ryall et al. 1995; Doyle et al. 1991).

## **1.6 CITRUS FRUITS AND JUICES**

Dietary citrate has been shown to play an important role in urolithiasis. Citrus fruits and juices represent a naturally rich source of citrate and provide a dietary citrate load equivalent to that of citrate supplements (Seltzer et al.1996). Such fruits include lemon, orange, grapefruits and lime (Wabner and Pak 1993; Seltzer et al. 1996; Trinchieri et al. 2002; Tosukhowong et al. 2008). These citrus fruits/juices may be important in the treatment of hypocitraturia and the management of CaOx or CaP stone patients, who may not be able to tolerate commercial therapy which requires the ingestion of several tablets or liquid supplements daily or who cannot bear the inherent financial burden associated with these preparations (Seltzer et al.1996).

As mentioned previously, citrus fruits/juices are a well-known natural source of citrate and therefore could represent an alternative to pharmacological citrate therapy. Several studies have investigated the impact of citrus juices on urinary parameters and have indeed shown that they have a positive effect towards hypocitraturia and CaOx crystal formation.

The advantage of citrus juices is that they are better tolerated and they are less cost effective than pharmacological treatments. In addition to this, some reports have shown the dropout rates of K-citrate due to the inconvenience of multiple administrations and the gastrointestinal intolerance (Pak et al. 1985; Schwille et al.1992; Barcelo et al. 1993, Hofbauer et al. 1994). This is supported by a recent review by Mattle and Hess (2005) which stated that up to 48 % of alkali citrate-treated patients withdrew prematurely from the studies because of intolerable side effects.

### **1.6.1 Grapefruits, Orange, Blackcurrant and Cranberry juices**

Trinchieri et al. (2002) showed that the ingestion of grapefruit juice in healthy subjects caused an increase in urinary citrate, calcium and magnesium excretion. In another study, Hönow et al. (2003) showed that apart from an increase in urinary citrate excretion, urinary pH also increased after the ingestion of grapefruit juice and hence the RS of CaOx decreased. Orange juice has also been investigated (Wabner and Pak 1993; Hönow et al. 2003; Odvina 2006). These studies have shown that orange juice is capable of

increasing urinary pH and citrate excretion. Similar results have been reported for blackcurrant juice (Keßler et al. 2002).

In all of these studies, urinary citrate excretion was increased. It is because of these results that investigators are convinced that citrus fruits can be used as a pharmacological therapy.

However, not all of the studies have produced entirely favourable results. Goldfarb and Asplin (2001) in their study showed that the ingestion of 240 ml of grapefruit juice 3 times a day by healthy subjects resulted in an increase in citrate excretion but that urinary oxalate also increased. Furthermore, in two studies involving cranberry juice, urinary pH decreased, there was no affect on urinary citrate excretion, while urinary calcium and oxalate increased thereby increasing urinary saturation of CaOx and uric acid (Keßler et al. 2002; Gettman et al. 2005). However, the acidification of urine by cranberry juice was suggested to be useful in the treatment of brushite and struvite stones as well as in the treatment of urinary tract infections.

The unfavourable results reported by Keßler et al. and Gettman et al. are in contrast to those reported by McHarg et al. (2003) who showed that cranberry juice favourably altered 3 key urinary risk factors namely, oxalate and phosphate excretion (which decreased) and urinary citrate (which increased). It is therefore apparent that the potential therapeutic value of the above-mentioned group of juices has not been convincingly demonstrated.

### **1.6.2 Lemon juice**

Several studies have investigated the effects of lemon juice in stone forming patients (Seltzer et al. 1996; Koff et al. 2007; Kang et al. 2007; Penniston et al. 2007; Aras et al. 2008). Lemons contain high concentrations of citric acid (Seltzer et al. 1996). Thus, a half cup of pure lemon juice can provide a daily amount of citrate comparable to that of pharmacological therapy (Seltzer et al. 1996). The above mentioned studies (Seltzer et al. 1996; Kang et al. 2007; Penniston et al. 2007; Aras et al. 2008) showed that the ingestion of lemon juice increases the urinary excretion of citrate, while that of Koff et al. (2007) showed that lemon juice did not provide improvements in urinary citrate or pH.

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They also showed increased urinary pH and decreased calcium levels. Because of the improvement in the urinary parameters of citrate and/or pH after the ingestion of lemon juice in either healthy subjects or kidney stone patients, it can be concluded that lemon juice may be of great importance in the treatment of urolithiasis.

### **1.6.3 Lime juice**

Lime (*citrus aurantifolia*), is a wild species originally from the Rutaceae family and it is common in Southeast Asian countries (Tosukhowong et al. 2008). It is rich in citrate as well as various antioxidants such as ascorbic acid, polyphenols and flavonoids (Gharagozloo et al. 2002). It is inexpensive and is used as a routine cooking ingredient, fruit, juice, preserved snack and as a medicinal agent (Wagner and Wu 2002; Xu et al. 2003; Bailey et al. 2003).

The effect of lime juice consumption on urinary pH was investigated by Mazdak et al. (2006). It was found that urinary pH increased. The authors suggested that its consumption can support treatment and prevention of uric acid and cystine stones as well as management of gout due to its alkalizing effect.

Tosukhowong et al. (2008) investigated the effects of lime powder ingestion in CaOx nephrolithiasis patients. They found a significant elevation of urinary citrate, potassium and pH while calcium and oxalate were unaltered. These results showed that lime delivers an antilithogenic action for treating nephrolithiasis that is equivalent to that of standard potassium citrate.

## **1.7 UROLITHIASIS IN SOUTH AFRICA**

As mentioned before, the incidence of kidney stones in the South African black population is extremely rare compared to the white population. Even though there has been a slight increase in the incidence of kidney stones in urban blacks in recent years, it still occurs in less than 1 % of this group, but in 12-15 % of the white population (Muskat 1951; Modlin 1967; Pantanowitz et al. 1973; Meyers et al. 1994; Whalley et al. 1998). The prevalence of stones in whites is similar to those in Western countries (Modlin 1967; Whalley et al. 1998).

Factors such as those discussed earlier in this chapter, as well as others, are likely to contribute to the rarity of stones in the black South African population group.

### 1.7.1 Urine chemistry

Several studies have compared urine composition data of South African black and white subjects in order to identify differences which might account for the rarity of stones in the former group (Modlin 1967; Meyers et al. 1994; Whalley et al. 1998; Lewandowski et al. 2005).

Urinary calcium has been consistently reported as being lower in black subjects compared to whites (Modlin 1967; Meyers et al. 1994; Whalley et al. 1998; Rodgers and Lewandowski 2002; Charlton et al. 2005). Urinary citrate excretion has been reported as being lower in the black group (Modlin 1967; Meyers et al. 1994; Whalley et al. 1998; Lewandowski et al. 2001). Reports have also shown that urinary sodium excretion in black subjects is higher than that in whites (Modlin 1967; Meyers et al. 1994).

Urinary pH and phosphate have been reported to be lower in blacks (Modlin 1967; Lewandowski et al. 2001). Intriguingly, no differences in urinary oxalate have been reported in most of these studies. However one study found this variable to be higher in the black group (Lewandowski et al. 2001). Some of these results are surprising and counterintuitive (lower citrate, lower pH, higher oxalate) because they suggest that black subjects have a *higher* risk of CaOx stone formation than white subjects.

On the other hand, while lower urinary calcium and phosphate levels occur in black subjects, they nevertheless lie within the *normal range*. Thus, while these differences could account for a lower prevalence of stone disease in the black group, they cannot account for the absence of the disease.

### 1.7.2 Diet

Studies in the Kidney Stone Laboratory at the University of Cape Town as well as those by others have shown that the South African black population has a dietary intake that is high in oxalate, (because of regular intake of spinach) (Viljoen and Gericke 2001), low in

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calcium because of lactose intolerance (Viljoen and Gericke 2001; Charlton et al. 2005), low in magnesium (Whalley et al. 1998; Charlton et al. 2005) and low in vitamin B<sub>6</sub> (Lewandowski et al. 2001).

As with urine composition studies, these results are surprising and counterintuitive as all of these eating habits are risk factors for hyperoxaluria. However, notwithstanding these, urinary oxalate in this race group has been shown to be within the normal range (Whalley et al. 1998; Rodgers and Lewandowski 2002; Lewandowski et al. 2005).

On the other hand, investigators have shown that South African blacks have a diet that is high in sodium compared to whites (Modlin 1967; Meyers et al. 1994; Whalley et al. 1998). As mentioned before about dietary sodium, hypercalcuria is an important lithogenic consequence of excessive intake of dietary salt and this increases the risk of CaOx stone formation (Pak 2004). It is therefore somewhat surprising that urinary excretion of calcium in blacks has been found to be lower. In order to explain these (and other) dietary anomalies, it has been suggested that the South African black group may have different mechanisms for handling lithogenic agents than the white population group (Lewandowski et al. 2001; Rodgers and Lewandowski 2002).

### **1.7.3 Proteins and microbiological activities**

Urinary proteins are well known as macromolecular inhibitors of urolithiasis. Studies at the Kidney Stone Research Laboratory (University of Cape Town) have investigated the inhibitory role of several urinary proteins in the two race groups. These include urinary prothrombin fragment 1 (UPTF1) (Webber et al. 2002), Tamm-Horsfall mucoprotein (THM) (Craig et al. 1999, 2001) and Albumin (Rodgers et al. 2006).

Studies on UPTF1 showed that it is an inhibitor of CaOx crystallization and that the protein isolated from the urine of blacks was superior to that obtained from whites in this regard (Webber et al. 2002). The authors suggested that the urine composition of blacks may influence their UPTF1 conformation in such a way that it performs better as an inhibitor than when it is in the urine milieu of white subjects (Webber et al. 2002).

Similarly, THM protein was isolated from the urine of both race groups and was found that the protein from black subjects was a stronger inhibitor of CaOx crystal aggregation than that obtained from white subjects (Craig et al. 1999, 2001).

Finally, in another study, albumin was also isolated from both groups and found to be a superior inhibitor of CaOx crystal growth and aggregation in black subjects (Rodgers et al. 2006).

Another factor which is of interest is the gastrointestinal absorption of oxalate which was measured by Lewandowski et al. (2005). No difference between these two race groups was found despite the fact that blacks have a higher dietary intake of oxalate. This might be due to the lower endogenous synthesis of oxalate in blacks. The other possible explanation is that blacks may excrete more calcium into the intestine thereby stopping more gastrointestinal oxalate being bound and thus excreted via the faeces (Lewandowski et al. 2005). A more likely explanation is that South African blacks have greater colonization of oxalate-degrading bacteria (Lewandowski PhD Thesis, 2003) than whites, hence less oxalate available for gastrointestinal absorption and urinary oxalate.

## **OVERVIEW**

The empirical evidence presented in section 1.7 strongly suggests that the handling of lithogenic and antilithogenic agents by South African black subjects is different to that which occurs in their white compatriots. While there are many such agents which still remain to be investigated, lime powder was selected for study in the present MSc project with a view to exploring whether it too is handled differently in the two population groups and if so, whether these differences might contribute towards understanding why stone rarity occurs in the one group but not in the other.

### **1.8 OVERALL AIM AND OBJECTIVES**

The overall aim of the present study was to investigate the handling of lime powder ingestion in subjects from South Africa's two population groups, with a view to gaining insight into why the incidence of urolithiasis is different between them.

The individual objectives to achieve this overall aim were:

- (i) to measure the inhibitory activity of lime powder in artificial urine.
- (ii) to measure the relative inhibitory activity of lime powder after its *in vitro* introduction into the voided urine of healthy black and white South African subjects.
- (iii) to measure and compare urinary risk factors in the two race groups before and after the ingestion of lime powder.

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**CHAPTER TWO**  
**GENERAL METHODS**

## 2.1 INTRODUCTION

This chapter describes the methods that were used in the different studies undertaken for this dissertation.

Experiments commenced with the preparation of artificial urine (AU). The inhibitory effects of lime powder on CaOx crystallization *in vitro* were investigated using different crystallization experiments.

Also described in this chapter are the experiments and risk indices used to determine and/or measure the inhibitory activity of lime powder towards CaOx crystallization in the trial study.

## 2.2 METHODS

### 2.2.1 Manufacture of lime powder

Lime powder was obtained from Thailand as part of a collaborative agreement between the Kidney Stone Research Laboratory (University of Cape Town) and a research group at Chulalongkorn University, Thailand. The powder was derived from natural lime, *citrus aurantifolia*. Lime fruits from local orchards in the central region of Thailand were harvested and were then thoroughly washed, cut in half and squeezed with a hand-squeezer to yield fresh lime juice. The seeds were filtered out and the lime juice was processed to reduce bitterness (Yachantha et al. 2007; Tosukhowong et al. 2008). Methods describing how bitterness was reduced are not available.

The lime juice was then freeze-dried using a freeze dryer (Model FDS, Heto-Holten AS, Denmark). Its chemical composition is given in Table 2.1 (Yachantha et al. 2007). The composition and physical characteristics (colour, odour and flavour) resembled that of the original lime (Tosukhowong et al. 2008). Aluminium sachets containing five grams of lime powder were packed and completely sealed.

**Table 2.1:** Chemical composition of lime powder (Yachantha et al. 2007)

<i>Variables</i>	<i>Component mass conc. (mg/g)</i>
Citrate	818.4
Oxalate	0.052
Total Na	1.32
Total K	102.74
Total Mg	0.24
Total P	0.815
Total Ca	15.59
Ascorbic acid	27.30
Total polyphenols	9.20
Flavonoids	8.80

### 2.2.2 Preparation of Artificial Urine (AU)

Artificial urine (AU) was prepared according to the method of Walton et al. (2005). A standard solution of 320 mM of NaCl, 99.5 % (Orion Chemicals), 50 mM NaH<sub>2</sub>PO<sub>4</sub> 99.102 %, (Merck), 6.52 mM MgCl<sub>2</sub>, 99 % (Riedel-de Haën), 164.2 mM KCl, 99.5 % (Merck), 4.34 mM K<sub>3</sub>Citrate, 99 % (Merck), 43.8 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 99.5 % (Merck), 7.0 mM NH<sub>4</sub>Cl, 99.5 % (Merck) was prepared using distilled water in appropriate volume. The pH of the solution was adjusted to 6.0 using 5 M of NaOH and the solution was stored at 4 °C until it was ready to be used. The solution was warmed at 37°C and filtered (0.22 µm) immediately before use. Two solutions of 50mM Na<sub>2</sub>Ox, 99.5 % (Associated Chemical Enterprises c.c) and 120 mM CaCl<sub>2</sub>, 95 % (Merck) were prepared separately and filtered (0.22 µm). 1780 µl of the 120 mM CaCl<sub>2</sub> solution and 286 µl of the 50 mM Na<sub>2</sub>Ox solution were mixed to achieve final concentrations of 2.14 mM and 0.143mM for calcium and oxalate respectively.

### 2.2.3 Subjects, urine collection, treatment and physicochemical properties

South African black and white healthy males with no history of kidney stone formation participated in the *in vitro* and *clinical trial* studies described in this dissertation. Subjects were excluded if they were on any medication that would interfere with the studies and they were asked to stop taking their vitamin supplements three days before they commenced with each study.

24 hour urine samples were collected in plastic bottles without preservative from 5 black and 5 white healthy male subjects (age range 18-32 years) on their free unrestricted diets. Each sample was tested for haematuria and nitrite using urinalysis test strips (Medi Test Combi 5N, Macherey-Nagel; DÜren). Those that tested positive for either were discarded. All urine samples were analysed for pH, sodium, potassium, calcium, oxalate, urate, citrate, chloride, magnesium, phosphate and creatinine as described below.

pH was measured using a Microprocessor pH meter (Hanna Instruments). Sodium, potassium, magnesium and calcium were determined using atomic absorption spectroscopy (Varian 1275 Model; Australia) (Willis 1969, Trudeau and Freier 1967; Fernandez and Kahn 1971) while commercially available assay kits from Sigma Aldrich and Boehringer Mannheim were used for oxalate (Chiriboga 1963) and citrate, respectively (Gruber and Möllering 1966). Chloride was determined using a chloride sensitive electrode (Beckman Coulter Inc). Creatinine was determined using picric acid (Rock et al. 1986). Phosphate was determined using ammonium molybdate while urate was determined using uricase (Synchron LX assay kits, Beckman Coulter Inc) (Fossati et al. 1980).

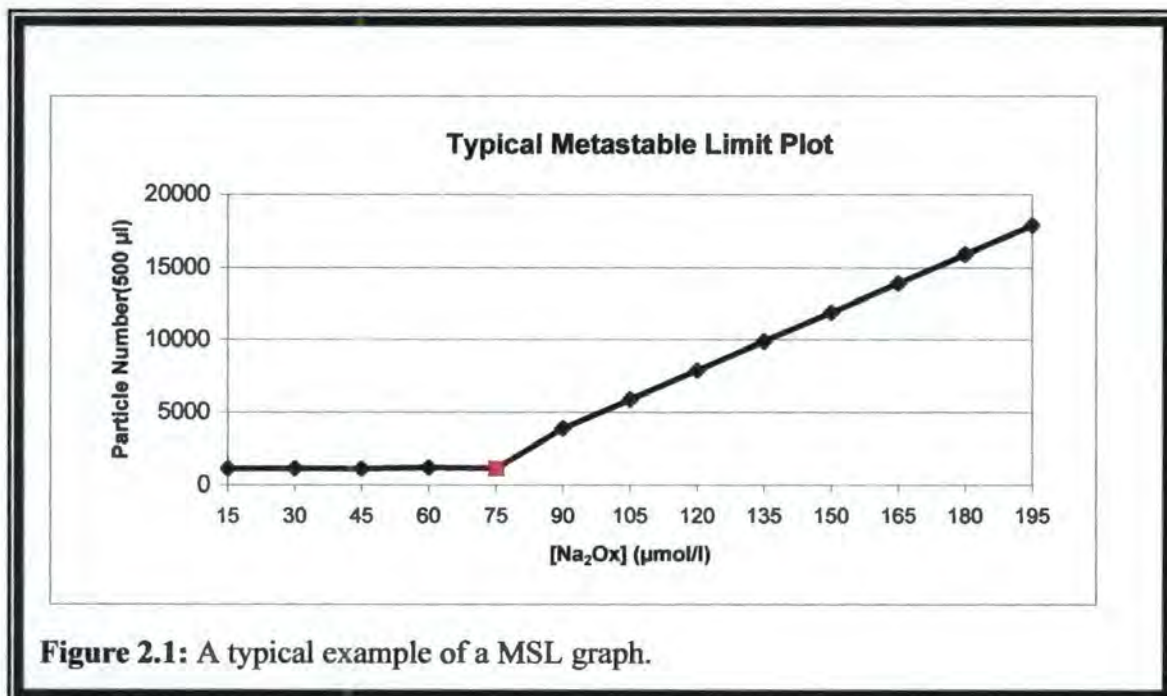
### 2.2.4 Crystallization Experiments

#### CaOx Metastable Limit (MSL)

Urine samples were prepared for determination of the CaOx metastable limit (MSL) by filtering successively through a 0.75 µm pre-filter (Macherey-Nigel; GmbH and Co., Germany) and 0.45 µm nitrocellulose filter (Sartorius AG, Germany). The MSL of each sample was determined following the method described by Ryall et al. (1985).

Thirteen aliquots (10 ml) of each sample were added to Coulter cups and incubated at 37°C in a shaking water bath at 100 rpm (Labmark, Johannesburg) for 10 minutes. Each aliquot was then successively dosed with 100 µl of sodium oxalate (Na<sub>2</sub>Ox) of increasing concentrations (15 mmol/L to 195 mmol/L) and incubated for a total of 30 minutes each. The particle number and volume in each aliquot were measured using a Coulter Multisizer II (Coulter Electronics Ltd, Luton, UK) fitted with a 140 µm orifice (2.8-90 µm particle size range).

The concentration of Na<sub>2</sub>Ox corresponding to the dose which caused a sudden increase in particle number was regarded as a measure of the MSL of the particular sample under investigation. A representative curve which shows the determination of the MSL is shown in Figure 2.1. The final Na<sub>2</sub>Ox concentration corresponding to the metastable limit is indicated by a red point.



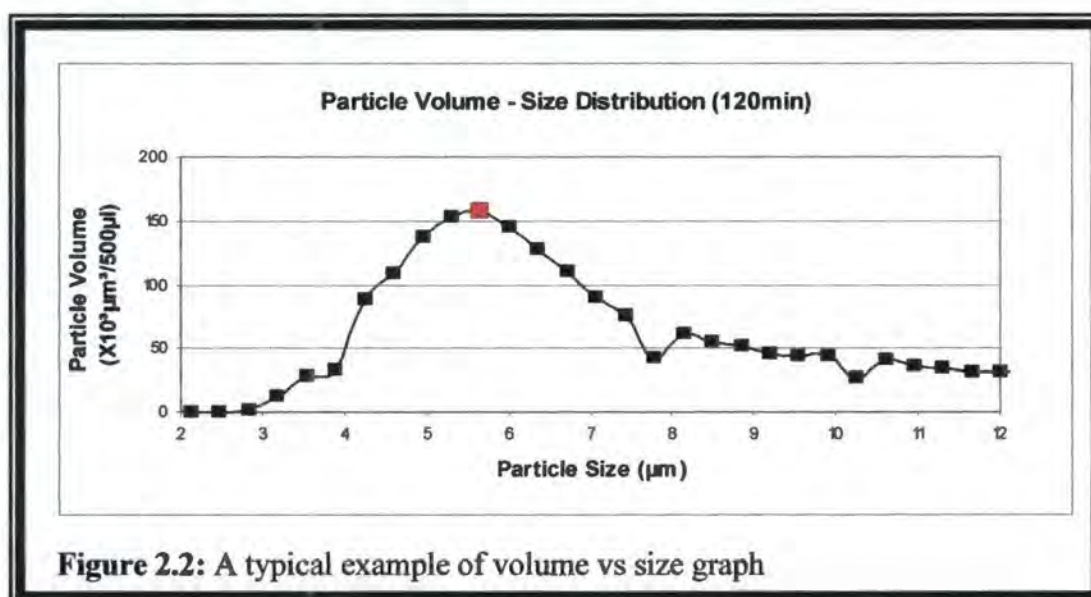
**Figure 2.1:** A typical example of a MSL graph.

### CaOx Particle Size-Volume Distributions (PSD)

Once the MSL had been determined, 50 ml of each urine sample was incubated in soda-lime bottles in a shaking water bath (Labmark, Johannesburg, 100 rpm) at 37 °C for 10

minutes. An aliquot of 10 % (v/v)  $\text{Na}_2\text{Ox}$  corresponding to 30 mmol/L above the previously determined MSL was then added to the sample to induce crystallization.

Thereafter the samples were incubated for two hours and the total volume of particles precipitated during the incubation period and the mean particle size were determined using the Coulter Multisizer II mentioned previously. Graphs of particle volume vs particle size were constructed. The mode of the curve represents the mean size of precipitated particles, while the total volume of precipitated particles is represented by the total area under the curve. A typical volume vs size graph is shown in Figure 2.2.



### Scanning Electron Microscopy (SEM)

All urine samples were dosed with aqueous  $\text{Na}_2\text{Ox}$  as described in the previous paragraph before being examined by scanning electron microscopy.

At the end of the 2 hour incubation period, 2 ml of the dosed urine was filtered through 0.22  $\mu\text{m}$  filters and dried at room temperature. Once the filter papers were dry, they were mounted on aluminium stubs using Sticky Tabs (ProSciTech, Thailand). The deposited crystals were viewed using a Leica S440 scanning electron microscopy (Leica Cambridge Ltd, England) operating at an accelerating voltage of 10 KV, a working distance of 10-15 mm and a probe current of 20-30 pA.

Micrographs for different sets of experiments were always recorded at the same magnification so that valid comparisons could be made, but the actual magnification varied for the different studies.

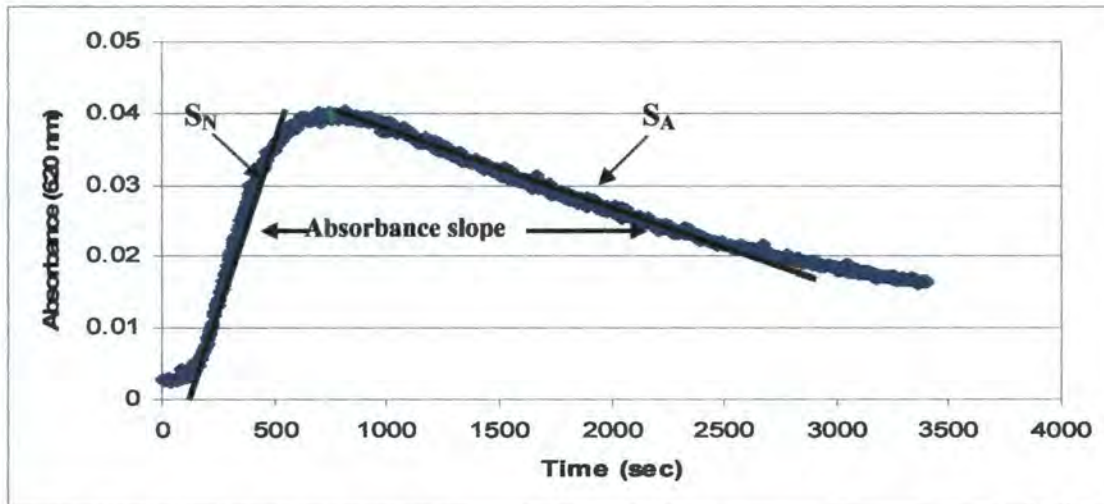
The procedure in which SEM was used to view crystals after the urine samples had been dosed was adopted because natural undosed samples in all experiments did not reveal crystals large enough to be visualised by SEM. Despite this exogenous method for producing crystalluria, comparisons of different samples in this way were regarded as being valid and appropriate, as all urines were treated in the same way.

#### **Simultaneous measurements of CaOx crystal nucleation and aggregation inhibition**

Crystal nucleation and aggregation were induced and monitored according to the procedure described by (Hess et al. 1995). Stock solutions of 8.5 mmol/L of CaCl<sub>2</sub> and Na<sub>2</sub>Ox (1.0 mmol/L) each containing 200 mmol/L and 10 mmol/L sodium acetate respectively, were prepared and the pH was adjusted to pH 5.7 by using 5M NaOH. The stock solutions were filtered through a 0.22 µm filter to remove any debris that might interfere with the spectrophotometric measurements.

The experiment was performed in a circulating water bath at 37 °C. CaCl<sub>2</sub> solution (1 ml) was transferred into a 10-mm cuvette placed in a spectrophotometer (Analytikjena, Specord 40, German), regulated at 37 °C and constantly stirred at 500 rpm. 1 ml of the Na<sub>2</sub>Ox solution was added to the CaCl<sub>2</sub> solution, resulting in final assay concentrations of 4.25 mmol/L calcium and 0.5 mmol/L oxalate, respectively. The automated time course measurement of OD<sub>620</sub> was performed after the addition of Na<sub>2</sub>Ox solution to the CaCl<sub>2</sub> solution. These values were recorded every 0.5 seconds for 60 minutes.

The maximum increase in the slope of OD<sub>620</sub> with time is interpreted as representing the maximum rate of formation of new particles, i.e rate of crystal nucleation and is termed (S<sub>N</sub>), while the decrease in the slope of OD<sub>620</sub> with time is interpreted as representing crystal aggregation and is termed (S<sub>A</sub>) (Hess et al. 1995). A typical crystal nucleation and aggregation curve is shown in Figure 2.3.



**Figure 2.3:** A typical plot of absorbance vs. time for the determination of CaOx nucleation and aggregation assays.

The experiment was performed in (i) AU alone, (ii) real urine alone, (iii) AU + lime powder and (iv) real urine + lime powder. The urine samples were tested at a ratio of 800 $\mu$ l of CaCl<sub>2</sub>: 400  $\mu$ l of urine: 800  $\mu$ l of Na<sub>2</sub>Ox. OD<sub>620</sub> was recorded every 0.5 seconds over 60 minutes. This urine concentration of 400  $\mu$ l urine in 2 ml total solution achieved the highest inhibition of crystal aggregation in the experiments performed by Hess et al. (1989). The percentage inhibition of both nucleation and aggregation by the test sample were determined from the following equations (Hess et al. 1995).

$$\% \text{ Nucleation inhibition } (N_{in}) = [1 - T_s/T_{sc}] \times 100 \quad \% \text{ Aggregation inhibition } (A_{in}) = [1 - T_s/T_{sc}] \times 100$$

$T_s$  = turbidity slope of plot of OD<sub>620</sub> vs. time in the presence of test sample.

$T_{sc}$  = turbidity slope of plot of OD<sub>620</sub> vs. time of the control without test sample.

### Preparation of calcium oxalate monohydrate (COM) crystals for growth assay

COM crystals were prepared following the method of Pak et al. (1975). Equal volumes of 10 mmol/L  $\text{CaCl}_2$  and 10 mmol/L  $\text{Na}_2\text{Ox}$  solutions were mixed at a constant rate of 1 ml/min using a peristaltic pump (Gilson, France). The mixture was then stirred at 4 °C for 7 days. Thereafter, the crystals were filtered through 0.22  $\mu\text{m}$  filter paper, washed with distilled water followed by methanol and then dried at 37 °C for one hour.

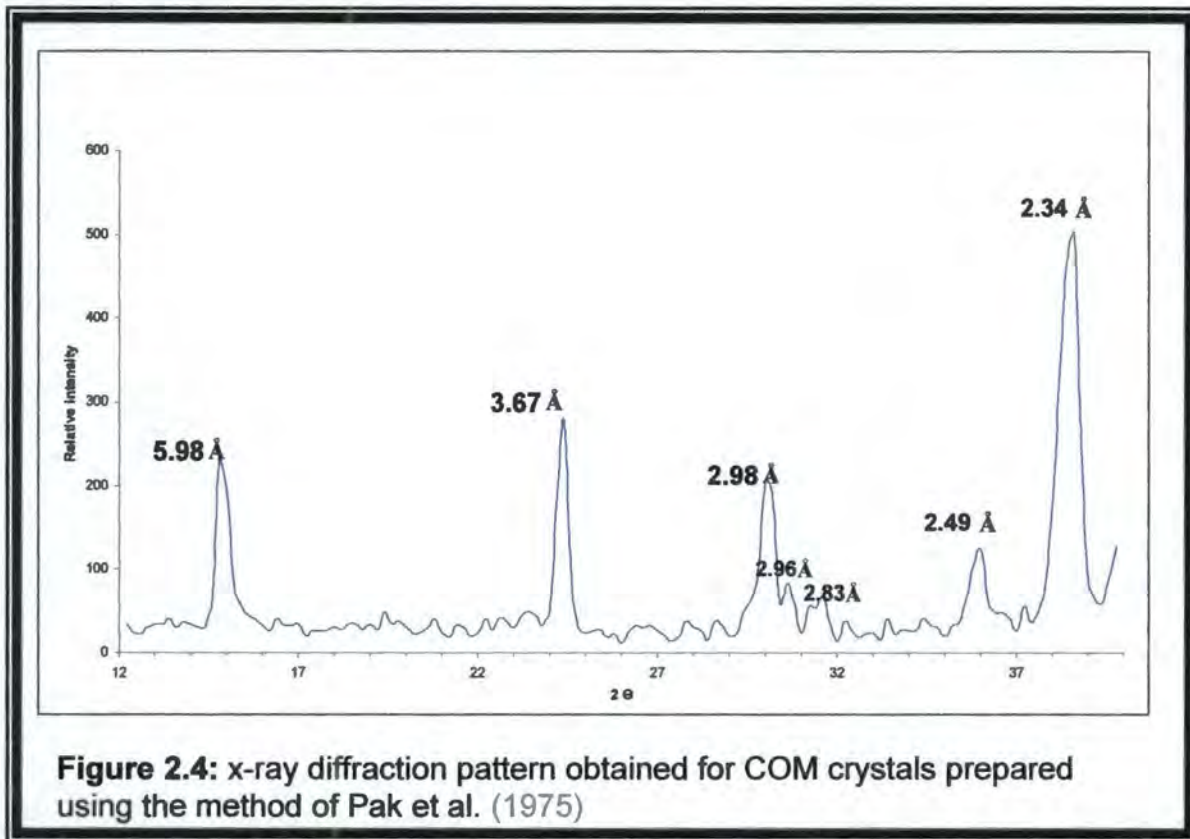
### Characterization of COM crystals

The COM crystals were characterized using X-ray powder diffraction (XRD). Powdered crystals were packed into aluminium trays and X-ray diffraction patterns were recorded using a Phillips PW 1050/25 vertical goniometer with  $\text{CuK}\alpha$  radiation of wavelength 1.5418 Å produced at 40 kV and 25 mA. The observed peaks were assigned by referring to the standard interplanar spacing and relative intensities for COM crystals (Sutor and Scheidt 1968) shown in Table 2.2. Relative intensities were compared qualitatively in the present study; quantitative measurements were not made.

**Table 2.2:** Interplanar spacing and relative intensities of COM powder pattern.

Standard d-spacing and relative Intensity data for COM		Present study
<i>d</i> -spacing (Å)	Relative intensity	<i>d</i> -spacing (Å)
5.93	100	5.98
5.79	25	
4.64	7	
4.52	6	
3.78	13	
3.76		
3.65	100	3.67
3.00	10	
2.97	46	2.98
2.91	12	2.96
2.89	10	
2.84	14	2.83
2.51	2	
2.48	30	2.49
2.41	5	
2.37	2	
2.34	90	2.33

Figure 2.4 shows the x-ray diffraction patterns obtained for COM crystals prepared using the method by Pak et al. (1975). Comparison with the standard x-ray diffraction data given in Table 2.2 confirms that the crystals are COM.



**Figure 2.4:** x-ray diffraction pattern obtained for COM crystals prepared using the method of Pak et al. (1975)

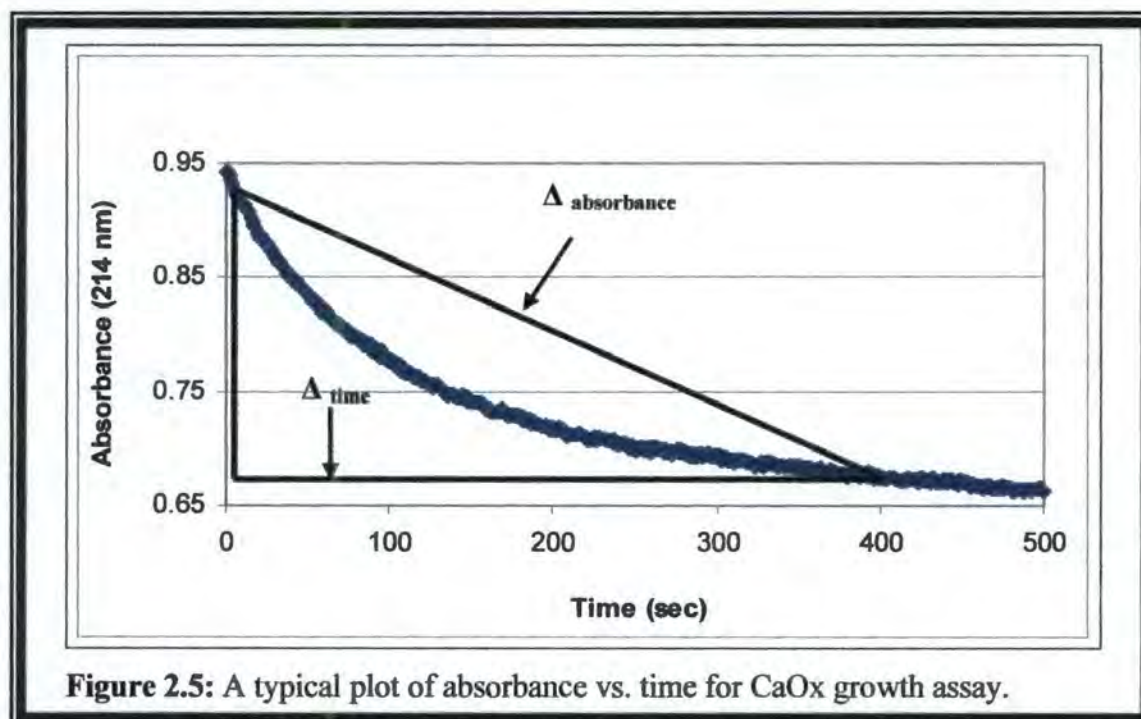
#### Preparation of COM crystal slurry for growth assay

A Tris buffer was prepared by mixing 10mM tris (hydroxymethyl)-aminomethane with 90 mM NaCl and the solution was adjusted to pH 7.2 using 5M NaOH. The tris buffer was filtered (0.22  $\mu$ m) before use. A seed crystal slurry was prepared by suspending the previously prepared COM crystals, in the same Tris buffer (16 mg/ml) and then allowing it to equilibrate overnight with fast stirring.

#### CaOx crystal growth Assay

The growth assay experiment was performed according to the method of Webber et al. (2007). A metastable solution of CaOx containing 0.5 mM CaCl<sub>2</sub> and 0.5 mM Na<sub>2</sub>Ox was prepared in Tris buffer (which was described previously).

The solution was maintained at room temperature with fast stirring throughout the experimental period. If the solution turned cloudy, a fresh solution was prepared. After the seed crystal slurry was equilibrated overnight, a 2 ml aliquot of the metastable CaOx solution was transferred to a quartz cuvette which was placed in a spectrophotometer (Specord 40) and stirred at 500 rpm. Growth was induced by the addition of the previously prepared seed crystal slurry (50  $\mu$ l). Absorbance was monitored at 214 nm and 37  $^{\circ}$ C for 30 minutes. Figure 2.5 shows a typical plot obtained for the crystal growth assay.



The experiment was performed for (i) AU alone, (ii) real urine alone, (iii) AU + lime powder and (iv) real urine + lime powder. AU samples were tested at a ratio of 1600  $\mu$ l metastable solution of CaOx: 400  $\mu$ l urine (AU): 50  $\mu$ l COM slurry. On the other hand, for real urine samples, this ratio failed to produce detectable crystallization. Various ratios were tested. Finally a ratio of 1990  $\mu$ l metastable limit solution of CaOx: 10  $\mu$ l real urine and 50  $\mu$ l COM slurry was used to determine the growth of the crystals. Inhibition of growth ( $I_G$ ) was calculated using the equation:

$$\text{Inhibition growth (I}_G\text{)} = [(A_C - A_L) / A_C] \times 100$$

where,

$$A_C = \frac{\Delta \text{ absorbance}}{\Delta \text{ time (400 sec)}} \quad \text{of the control}$$

$$A_L = \frac{\Delta \text{ absorbance}}{\Delta \text{ time (400 sec)}} \quad \text{of lime-dosed samples}$$

### 2.2.5 BONN Risk Index (BRI)

BRI is an experimentally determined ratio of the free ionized calcium concentration  $[\text{Ca}^{2+}]$  to the quantity of ammonium oxalate required to induce spontaneous crystallization of CaOx in the urine sample under investigation (Laube et al. 2004). BRI values  $< 1$  denote that samples are not at risk for kidney stone disease while values  $> 1$  indicate that samples are at risk.

Therefore,

$$\text{BRI} = \frac{[\text{Ca}^{2+}]}{[\text{Ox}^{2-}]} = \frac{\text{free ionised calcium (mmol/l)}}{\text{quantity of ammonium oxalate required to induce crystallization (mmol/100 ml urine)}}$$

BRI was determined in both the *in vitro* and *in vivo* (clinical trial) studies (Chapter 4 and 5).

### 2.2.6 Tiselius Risk Index (TRI)

The Tiselius risk index expresses the biochemical risk of CaOx formation (Tiselius 1982).

The mathematical expression is given by

$$\frac{(\text{Ca/Cr})^{0.71} \times (\text{Ox/Cr})}{(\text{Mg/Cr})^{0.14} \times (\text{Cit/Cr})^{0.10}}$$

where Ca, Ox, Mg, and Cit are the urinary excretion (mmol per 24 hour) of calcium, oxalate, magnesium and citrate, respectively and Cr is the urinary excretion (mol per 24 hour) of creatinine. TRI was determined in the clinical trial study only (Chapter 5).

### **2.2.7 Relative supersaturation (RS)**

The relative supersaturation values of CaOx (COM and COD), calcium phosphate (brushite) and uric acid were calculated by the computer program EQUIL 1.51b using urine composition data (Ackermann et al. 1989; Brown et al. 1994).

These relative supersaturations were determined in the clinical trial study (Chapter 5) only.

### **2.2.8 Statistical analysis**

Mean values and standard errors of the means of all data were calculated using analysis of variance (SAS statistical package, Statistica 8). For all the data, on the reasonable assumption that the populations had the same variance, these variances were pooled and a single standard error was obtained. Results were considered statistically significant if  $p \leq 0.05$ .

Mean particle size distributions were also calculated. Skew-normal distributions were fitted to each mean data set using the software package R (Azzalini and Dalla Valle 1999, 2003) and the parameter values for the location, scale and shape were determined. These values were used to compute theoretical moments and particle modes.

### **2.2.9 Ethics**

The project described in this dissertation was approved by the Ethics and Research Committee of the University of Cape Town.

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**CHAPTER THREE**  
**EFFECTS OF LIME POWDER**  
**SOLUTIONS ON CALCIUM OXALATE**  
**CRYSTALLIZATION IN ARTIFICIAL**  
**URINE**

### **3.1 INTRODUCTION**

As stated in Chapter 1, citrus fruits/juices are a known natural source of dietary citrate. Citrate is a well known inhibitor of CaOx crystallization. The presence of citrate in urine decreases the saturation of CaOx and CaP by forming complexes with calcium. It also inhibits crystal nucleation, growth and aggregation (Ryall et al. 1981; Pak et al. 1985; Pak 1987; Grases et al. 1989; Grases and Costa-Bauzá 1990; Conte et al. 1990; Tiselius et al. 1993; Laube et al. 2002).

Lime is rich in citrate and therefore is expected to act as an inhibitor of CaOx crystallization. The investigation of lime is the focus of this dissertation. As a starting point, its potential inhibitory property towards CaOx crystallization in AU was tested. This chapter describes experiments which address aim (i) (page 27).

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Study Design**

The lime powder was manufactured and prepared as described in Chapter 2.2.1 (page 50-51). Solutions of different concentrations of lime powder were prepared using distilled water and were modelled on a study by Yachantha et al. 2007. The different concentrations of lime powder were 0.125, 0.250, 0.500 and 1.00 mg/ml. If the solutions turned cloudy, they were discarded and a new solution was prepared. The pH of the lime solutions were not measured

### **3.2.2 Experimental methods**

The AU used for this study was prepared as described in Chapter 2.2.2 (page 51).

#### ***Crystallization experiments***

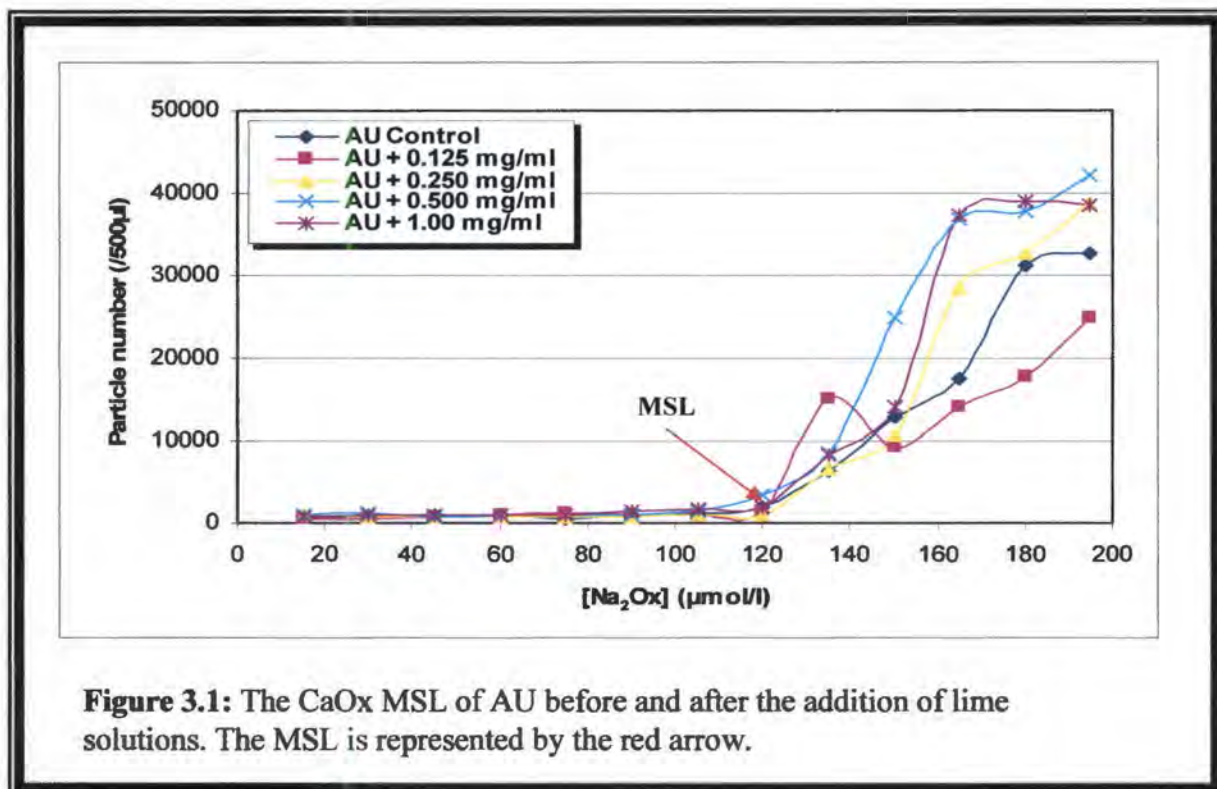
The CaOx metastable limit, particle volume-size distribution, scanning electron microscopy, crystal nucleation and aggregation inhibition, and CaOx crystal growth assay were determined in a control AU samples and in sample dosed with lime solutions of different concentrations. All these experiments were performed in duplicate. The techniques have been described in Chapter 2.

### 3.3 RESULTS

#### 3.3.1 Crystallization Experiments

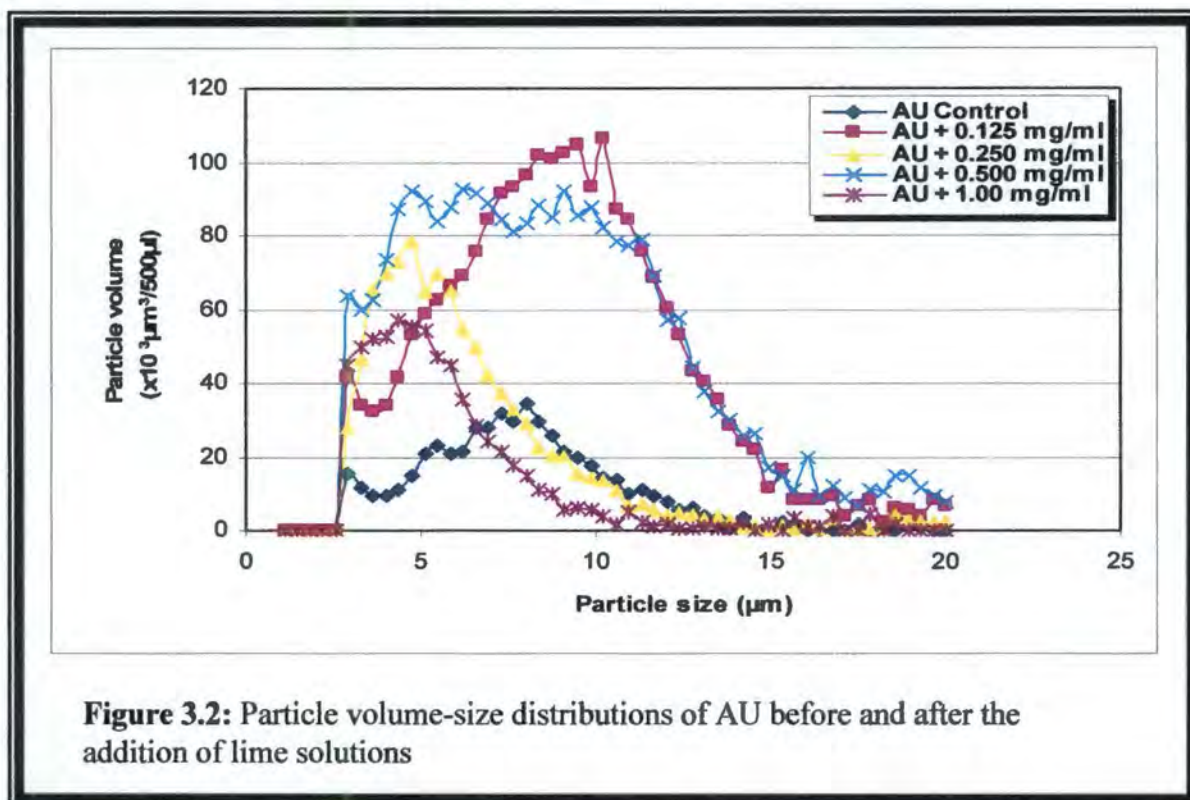
##### CaOx MSL

Graphs of particle number versus sodium oxalate concentration for the determination of CaOx MSL values in the different experiments are presented in Figure 3.1. These show that the MSL did not change as a function of the concentration of the lime solution and remained constant at a value of 120  $\mu\text{mol/l}$ . Data and plots of each sample are given in Appendix 1.



**CaOx PSD**

The mean PSDs of the control AU as well as those which had been dosed with lime solutions are presented in Figure 3.2. Quantitative values are given in Table 3.1. Plots of PSD experiments are given in Appendix 2.



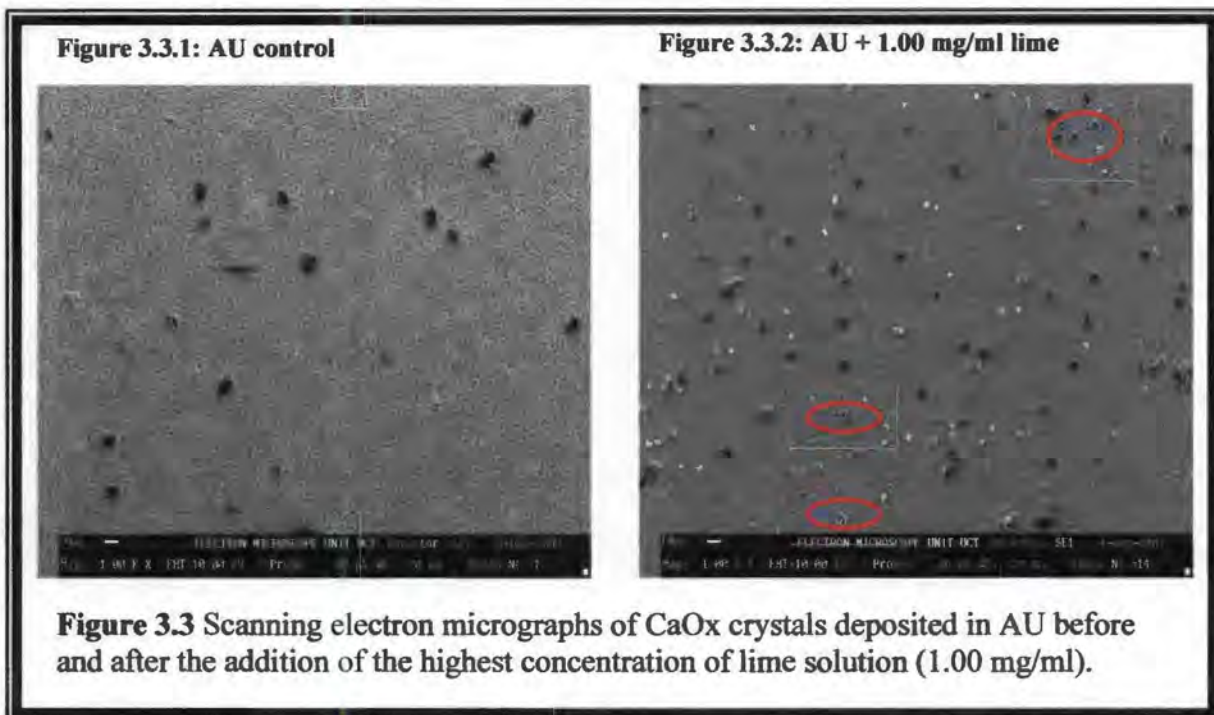
**Table 3.1:** Data derived from particle size distribution of AU dosed with different solutions of lime powder

<i>Samples</i>	<i>Particle size mode (μm)</i>	<i>Particle volume peaks (x10<sup>3</sup> μm<sup>3</sup>/500μl)</i>
AU control	7.18	34.4
AU + 0.125 mg/ml	7.70	104.6
AU + 0.250 mg/ml	4.09	78.5
AU + 0.500 mg/ml	3.21	92.3
AU + 1.00 mg/ml	3.08	57.3

Statistical analysis of these distributions showed that particle size decreased, while the particle volume increased as the concentration of the lime solution increased relative to the AU control.

### SEM

Figures 3.3.1 and 3.3.2 show SEM micrographs of CaOx crystals precipitated from AU before and after the addition of lime solution respectively. Comparison shows that crystal number increased while crystal size decreased. It is also noted that the nature of the calcium oxalate hydrate changed from only mono to a mixture of mono and dihydrate crystals (as indicated by red circle) after dosing with lime.

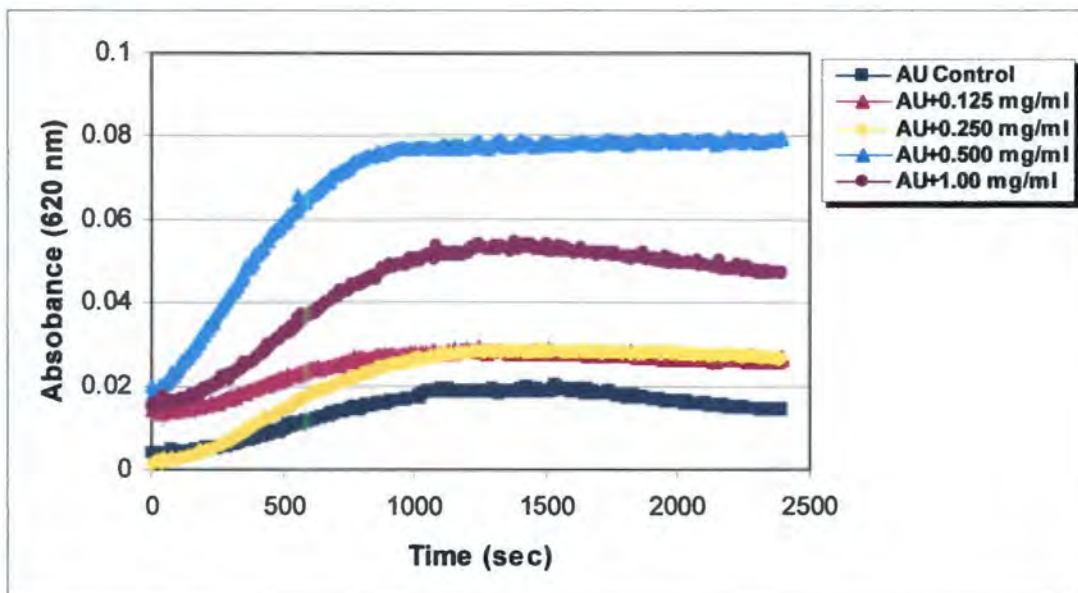


**CaOx crystal nucleation and aggregation**

The percentage inhibitions of both CaOx crystal nucleation and aggregation before and after the addition of different lime solutions are given in Table 3.2 and plots of absorbance vs time are shown in Figure 3.4.

**Table 3.2:** The percentage inhibition (% In) of both CaOx crystal nucleation and aggregation in the absence and presence of lime solutions.

<i>Samples</i>	<i>% In of Nucleation</i>	<i>% In of Aggregation</i>
AU control	95.4	85.4
AU + 0.125 mg/ml	93.9	92.8
AU + 0.250 mg/ml	92.2	97.8
AU + 0.500 mg/ml	76.6	98.5
AU + 1.00 mg/ml	87.7	98.9

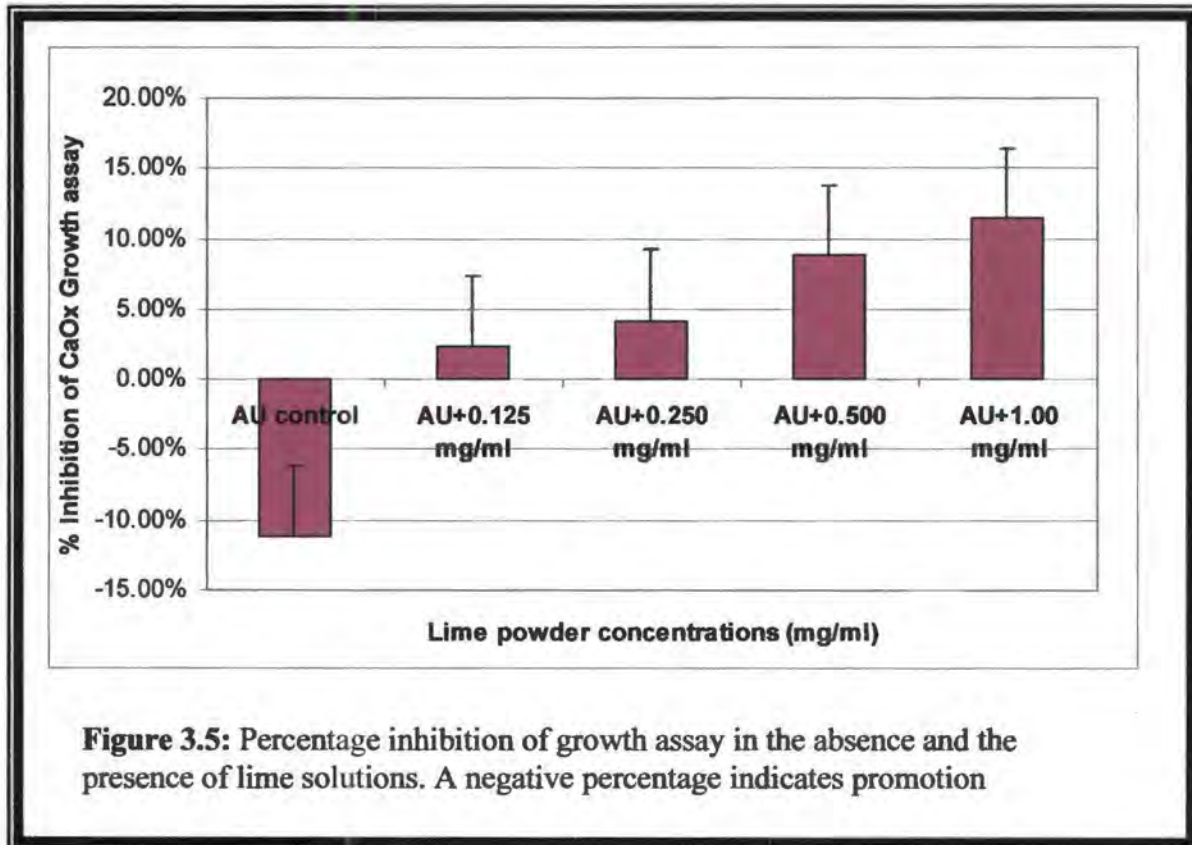


**Figure 3.4:** Plot of absorbance vs time for the simultaneous nucleation and aggregation assay before and after the addition of lime solutions

The percentage inhibition of nucleation showed a general decrease with increasing concentrations of lime solutions. On the other hand, inhibition of CaOx crystal aggregation increased consistently with increasing concentration of lime solutions. The individual plots of these experiments are given in Appendix 3.

**CaOx crystal growth**

The effect of lime on the % inhibition of crystal growth in AU is shown in Figure 3.5. Inhibition increased steadily and consistently as the concentrations of lime increased. The individual plots of these experiments are given in Appendix 3.



### 3.3.2 Summary

The results of the various crystallization experiments are summarised and briefly interpreted in Table 3.3.

**Table 3.3:** A summary of results and interpretation showing the effect of lime concentrations (0.125 mg/ml-1.00 mg/ml) in AU samples.

<i>Variables</i>		<i>Interpretation: Effect of lime</i>
MSL	no change	does not affect initiation of nucleation
PSD size	↓	inhibits growth
volume	↑	promotes growth or nucleation
SEM size	↓	inhibits growth
number	↑	promotes nucleation
% Inhibition of crystal nucleation	↓	promotes nucleation
% Inhibition of crystal aggregation	↑	inhibits aggregation
% Inhibition of crystal growth	↑	inhibits crystal growth

### 3.4 DISCUSSION

Since the CaOx MSL represents a measure of the ease or difficulty with which crystallization is initiated, the results of the present study have demonstrated that lime has no effect on this urinary property. However, once crystallization has commenced, the results show that lime does indeed have an effect on all three crystallization mechanisms. This conclusion can be drawn by consideration of the summary of results which are given in Table 3.3. It is observed that the total volume precipitated crystals in the PSDs, increased with increasing lime concentration. An increase in particle volume can arise as a result of an increase in crystal nucleation (more particles) or growth (bigger particles) or aggregation (in which the random packing of crystals creates pores which will ultimately increase the volume of the conglomerate). Promotion of growth can be ruled out by the results of the growth assay which demonstrated inhibition of this particular mechanism. In addition, growth inhibition was also demonstrated by the SEM observations. Similarly, promotion of aggregation can also be discounted, as inhibition of this particular mechanism was demonstrated in the aggregation assay.

Thus, promotion of nucleation remains the only possibility to account for the increase in particle volume. This is supported by the nucleation assay and by the SEM observation. Interestingly, nucleation has been interpreted as a favourable process as it provides a mechanism for rapidly decreasing saturation (Kavanagh 1992).

Mixture of COM and COD crystals were deposited after the addition of lime solutions compared to only COM crystals in the control sample (Figure 3.3.1). These results are favourable and of great importance. This is due to the report by Wesson et al. (1998) that COD crystals are less adherent to renal tubular cells. The other reason for the importance of COD deposition is that the higher positive charge on these crystals results in greater repulsive forces between crystals and therefore favours disaggregation (Webber 2003). This is indeed supported by the % inhibition of CaOx aggregation which showed a consistent increase after the addition of lime solutions.

It is noteworthy that the results which emerged in the present study in independent crystallization experiments are entirely consistent. These show that in AU, lime powder

promotes nucleation (PSD, SEM, inhibition of nucleation assay), inhibits growth (PSD, SEM, inhibition of growth assay) and inhibits aggregation.

Therefore, lime has been shown in the present study to have favourable effects in artificial urine on all three crystallization mechanisms and in the context of CaOx stone formation.

The results discussed in the next chapter will address whether similar effects occur in real urine.

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**CHAPTER FOUR**  
**IN VITRO EFFECTS OF LIME**  
**POWDER SOLUTIONS ON CALCIUM**  
**OXALATE CRYSTALLIZATION IN**  
**REAL URINE**

#### **4.1 INTRODUCTION**

In the previous section, the simple model of an artificial urine was used as the solution medium. Real urine is far more complex. Extension of the investigation to this medium is essential in order to establish the effects of lime solutions in humans.

Previous studies on the *in vitro* effects of fruit juices and natural extracts include that of Atmani and Khan (2000), Yachantha et al. (2007) and Kulaksizoğlu et al. (2008). These have shown that the citrus fruits are capable of inhibiting CaOx crystallization.

#### **OBJECTIVES**

The objective of this study was to investigate the effect of lime powder on CaOx crystallization in urine obtained from South African black and white subjects with a view to exploring whether:

- (i) similar results would be obtained to those obtained in AU
- (ii) different results might be obtained in the urine from the two race groups.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Study design

Two groups, consisting of 5 healthy South African black males and 5 healthy South African white males respectively, were age matched (ages 18-32). They were recruited from the undergraduate and postgraduate cohorts at University of Cape Town. None of the subjects in either group had a metabolic illness or any clinical problem and had no history of kidney stones.

For each subject, brief information about their social and medical history was collected as shown in Table 4.1. Details of the questionnaire are given in Appendix 4.

**Table 4.1:** Examples of the information requested about the subjects' social and medical history.

<i>Information Requested</i>	<i>Examples</i>
General	Name of subject, date of birth & date of participation in project
Nutritional/Exercise	Weight, height, allergic food, special diets, supplements, exercise
Exposure to toxic substances	Number of daily alcohol drinks
Medical history	Recent illness, current medication

The purpose and procedure of the study was explained to each subject and those who agreed to participate in the study signed a letter of consent (Appendix 4).

During the urine collection day (described on page 80), subjects were on a free diet but were advised to avoid certain foods according to Table 4.2 and to increase their fluid intake. Thus, subjects served as their own controls in accordance with the study by Karagülle et al. (2007). A 24-hour dietary recall questionnaire was used to record food and drinks on the day of urine collection. Food and drinks were analysed for macro- and micronutrients using the computer programme “Foodfinder” version 2 (Wolmarans et al. 2001).

**Table 4.2:** Examples of food that subjects were advised to avoid on the day of urine collection.

<i>Types of foods</i>	<i>Examples</i>
Oxalate-rich food	Spinach, rhubarb, beetroot, peanuts, cocoa, chocolate, tea, coffee
Salty food	Processed meats, snack foods, stock cubes, salty condiments and sauces, packed and tinned soups
Calcium-rich food	Fat-free milk, cheese, yoghurt

Different concentrations of lime solutions were prepared using distilled water as explained in Chapter 3.2. The concentrations which were used for this study were 0.125, 0.500 and 1.00 mg/ml. These concentrations were modelled from the ones used in the study by Yachantha et al. (2007). However, three out of four concentrations were used in this study as opposed to the AU study, due to experimental constraints.

#### ***4.2.2 Experimental methods***

##### ***Urine collection***

24 hour urine samples were collected in plastic bottles without preservative from each of the participants as explained in Chapter 2 (page 52).

##### ***Crystallization experiments***

The CaOx MSL, PSD, crystal nucleation and aggregation inhibition, and CaOx crystal growth assay were determined in control samples of all subjects (blacks and whites) and in samples dosed with lime solutions of different concentrations. The techniques have been described in Chapter 2.

##### ***Free calcium ion concentrations and BRI***

These parameters were determined as described in Chapter 2.2.5 (page 60). 2500  $\mu$ l of lime solutions ranging from 0.125 to 1.00 mg/ml was added to 100 ml of each urine sample under investigation. Measurements were taken before and after dosing the urines with lime.

##### ***Statistical analysis***

Statistical analyses were conducted as described in Chapter 2.2.8 (page 61).

## **4.3 RESULTS**

### ***4.3.1 Social and medical history***

Ten South African males participated in the study and were aged between 18-32 as mentioned on page 78. The study was conducted over 8 weeks. It was found that blacks had identical body mass index (BMI) to that of whites (23.03 vs 22.80) as shown in Table 4.3. On the other hand, white subjects participated in different physical exercises and they were exposed to alcohols (2-3 glasses) daily, while some black subjects were not involved in any exercises and most of them did not drink alcohol. None of the subjects were on special diet, had any illness and hence were not taking any medication prior the study.

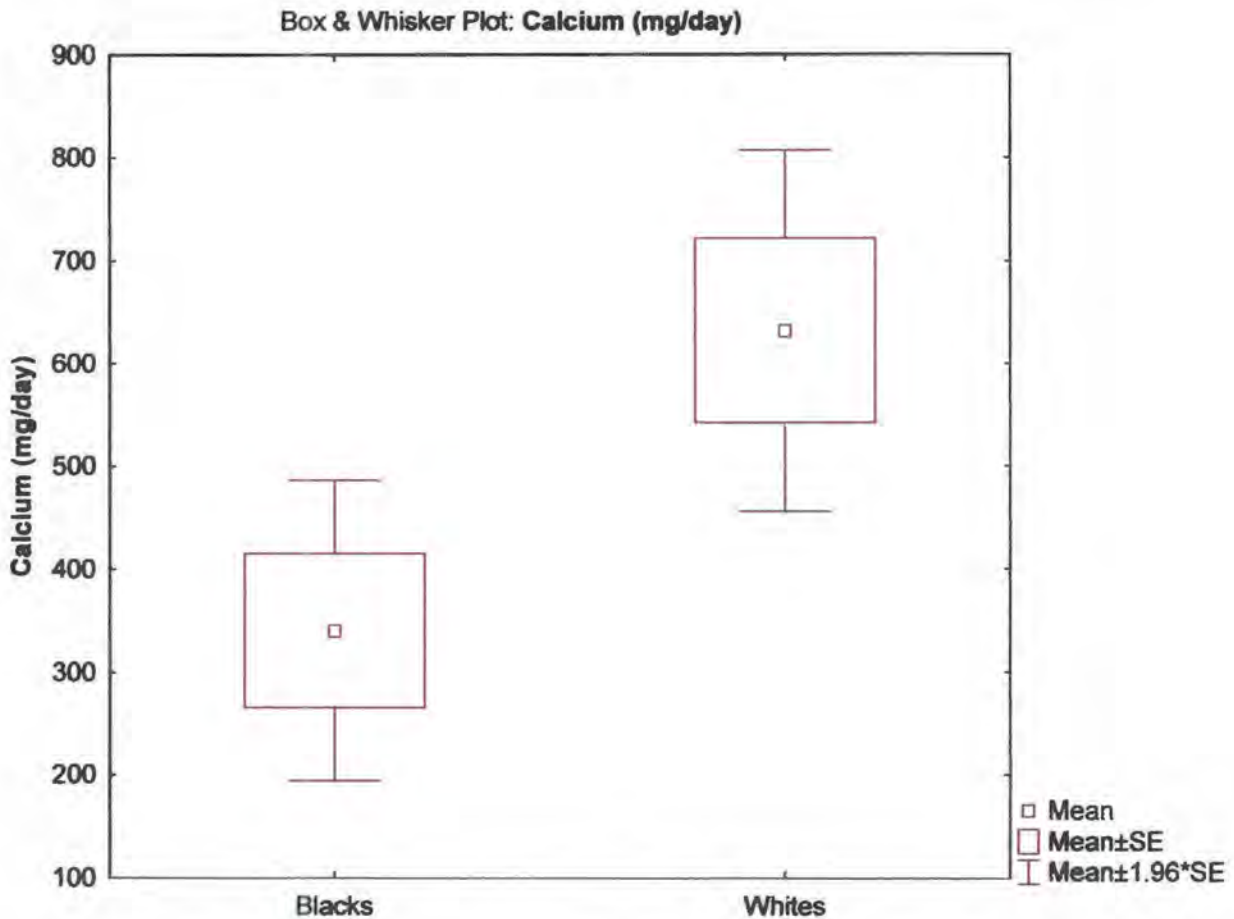
### ***4.3.2 Dietary analysis***

The mean intake of nutrients on the day of urine collection for the two race groups was determined from the completed dietary recall questionnaires. Values are given in Table 4.3. It is noted that only calcium was significantly different between the groups, namely the intake was lower in black subjects (340.4 mg/day vs 631.1 mg/day,  $p = 0.037$ ). This is depicted graphically in Figure 4.1. The daily nutrient intake of each subject is given in Appendix 5.

**Table 4.3:** Mean daily intake of nutrients for black and white subjects (SE)

<i>Variables</i>	<i>Blacks</i>	<i>Whites</i>	<i>P-values</i>
Moisture	2965.9 (536.5)	2053 (536.5)	0.263
Total Protein (g/day)	69.82 (9.46)	68.16 (9.46)	0.904
Animal Protein (g/day)	43.86 (7.39)	32.14 (7.39)	0.295
Total fat (g/day)	42.68 (9.64)	47.82 (9.64)	0.716
Carbohydrate (g/day)	185.48 (35.36)	163.74 (35.36)	0.675
Fibre (g/day)	18.78 (4.64)	24.12 (4.64)	0.439
Added sugar (g/day)	26.12 (4.45)	19.18 (4.45)	0.302
Oxalate (mg/day)	42.80 (23.68)	34.40 (23.68)	0.808
Ca (mg/day)	340.40 (82.41)	631.60 (82.41)	0.037*
Mg (mg/day)	243.40 (45.83)	289.60 (45.83)	0.496
Phosphate (mg/day)	950.40 (145.7)	1305.0 (145.7)	0.123
K (mg/day)	1570.40 (423.5)	2157.20 (423.5)	0.355
Na (mg/day)	1634.80 (219.9)	1842.40 (219.9)	0.523
Vitamin A (RE/day)	2912.00 (183.3)	6116.00 (183.3)	0.251
Vitamin B <sub>6</sub> (mg/day)	1.33 (0.381)	1.57 (0.381)	0.662
Vitamin C (mg/day)	76.60 (25.12)	65.00 (25.12)	0.836
Vitamin D (µg/day)	2.166 (0.829)	2.746 (0.829)	0.634
BMI	23.04 (1.174)	22.80 (1.174)	0.889

\*Significance at  $p < 0.05$

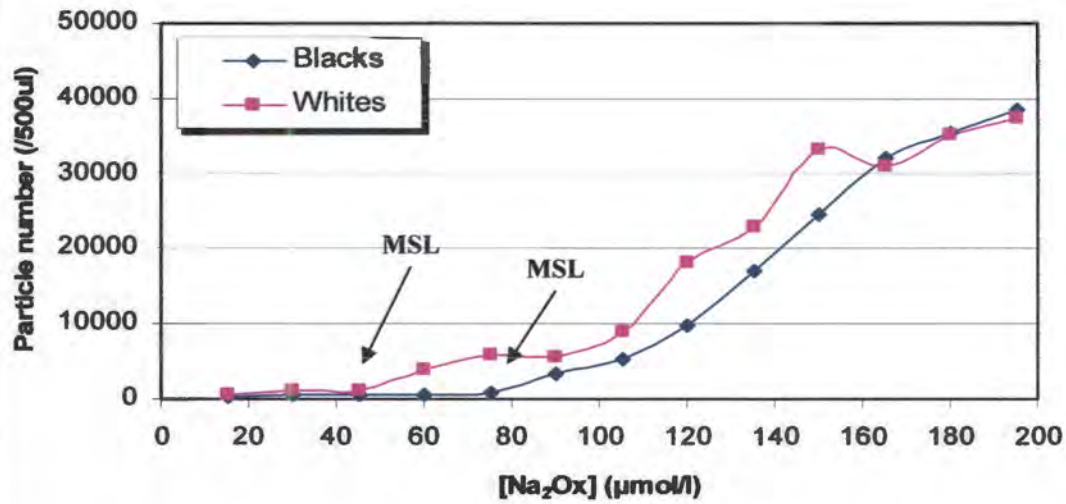


**Figure 4.1:** Comparison of the means of calcium intake for black and white subjects

### 4.3.3 Crystallization Experiments

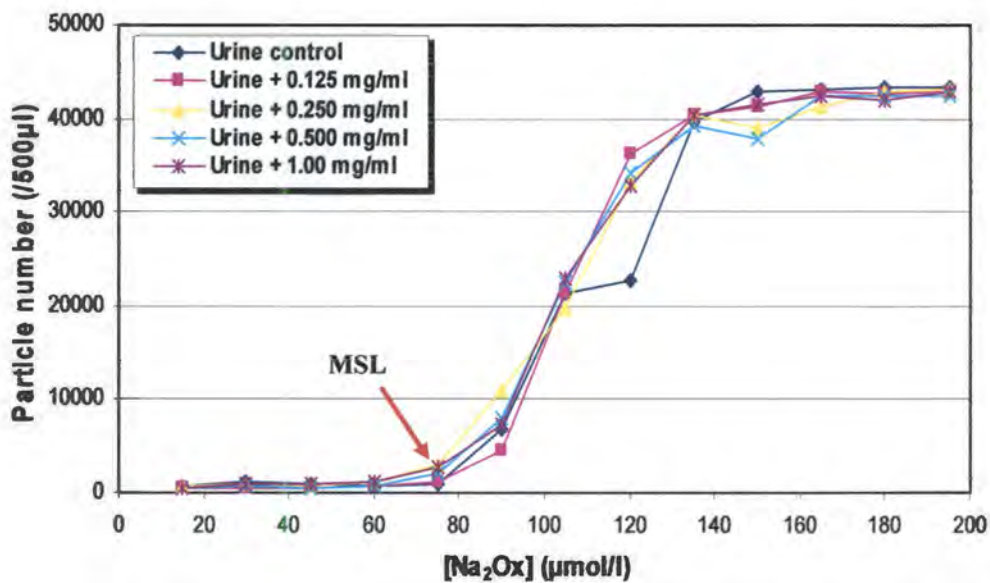
#### *CaOx MSL*

Figure 4.2 gives mean plots for the determination of the CaOx MSL in the control urines of 5 black subjects and 5 white subjects. These graphs show that the MSL in the urine of black subjects is higher than that in white subjects i.e. of 75 vs 45  $\mu\text{mol/l}$  respectively. The data and plots of each urine sample are shown in Appendix 1.



**Figure 4.2:** Plot of measured CaOx MSL of both black and white subjects (urine control)

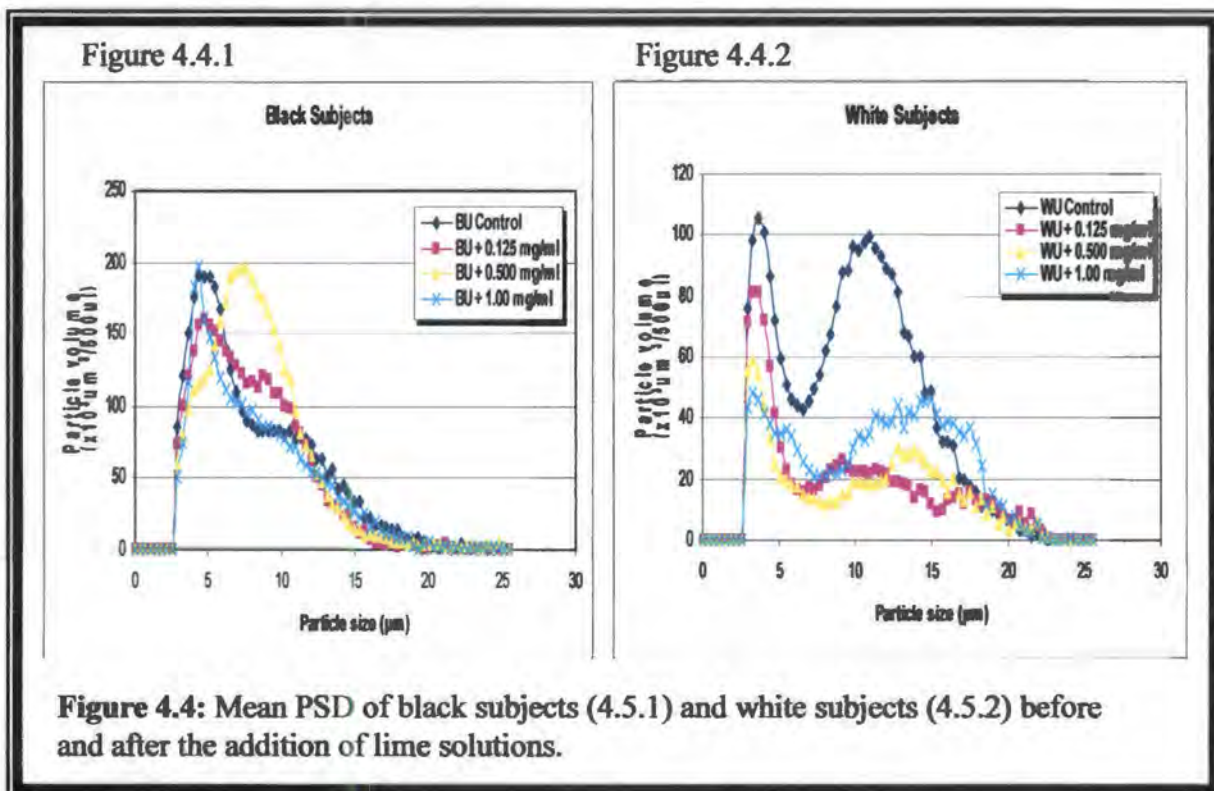
Within each group the CaOx MSL did not change after addition of lime solutions. This is demonstrated for black subjects in Figure 4.3.



**Figure 4.3:** The CaOx MSL of black subjects' before and after the addition of lime solutions. The MSL is represented by the red arrow.

**CaOx PSD**

Mean PSD plots for black and white subjects are shown in Figure 4.4 (1) and Figure 4.4 (2) respectively. Quantitative values for the mean particle sizes and particle volumes are given in Table 4.4. It is noted that mean sizes tended to decrease with increasing lime concentrations, albeit that there was a single outlier in both groups. Particle volumes of black subjects did not change with increasing concentrations of lime powder (except for the one outlier) while particle volumes of white subjects decreased with increasing lime concentrations. PSD plots for each subject are given in Appendix 2.



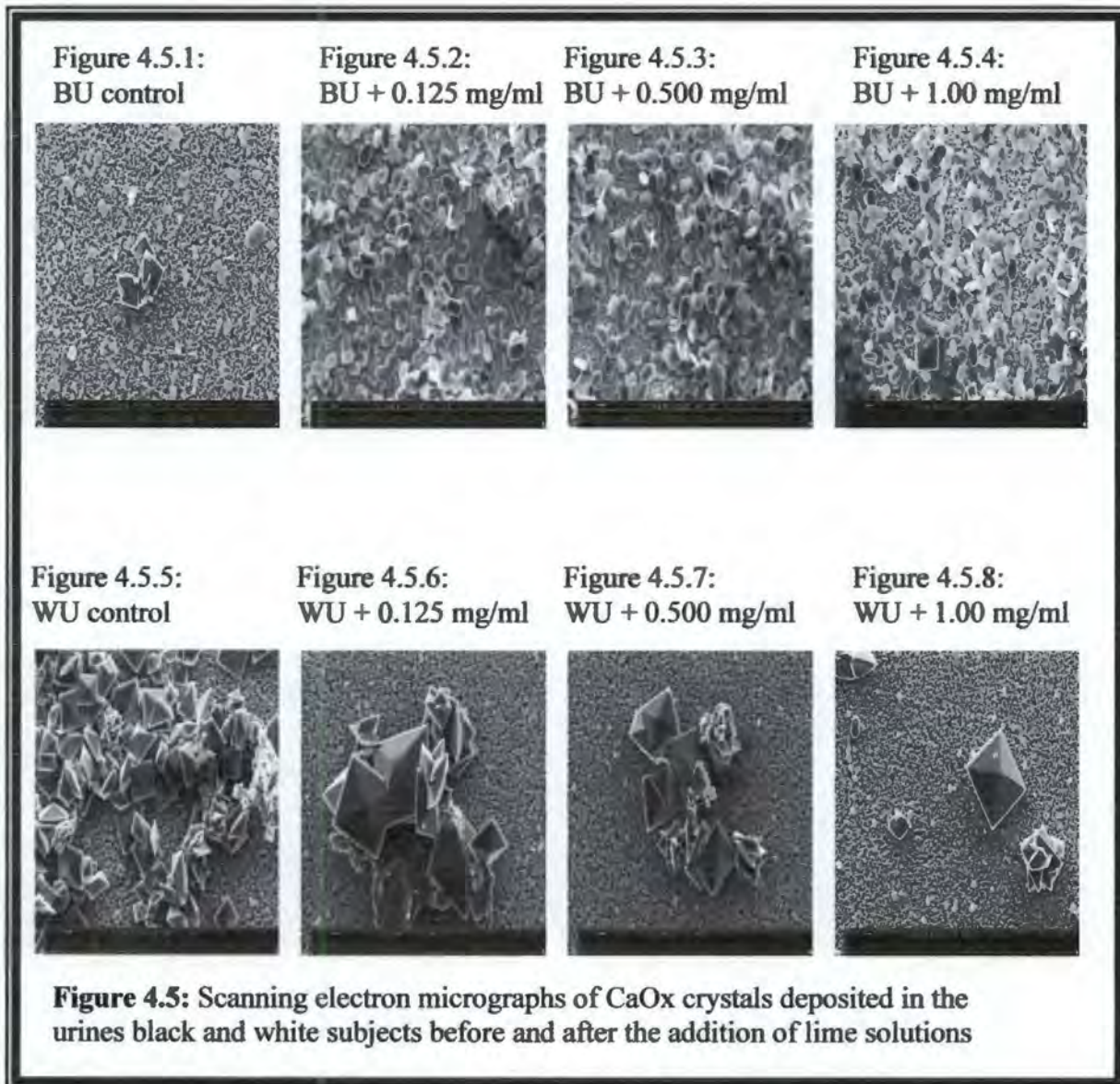
**Table 4.4:** Data derived from PSD of urines of black and white subjects before and after the addition of lime solutions.

<i>Samples</i>	<i>Particle size mode (<math>\mu\text{m}</math>)</i>	<i>Particle volume peaks (<math>\times 10^3 \mu\text{m}^3/500\mu\text{l}</math>)</i>
BU control	6.17	196.51
BU + 0.125 mg/ml	5.77	160.26
BU + 0.500 mg/ml	6.76	196.66
BU + 1.00 mg/ml	5.64	196.51
WU control	8.05	105.60
WU + 0.125 mg/ml	8.85	81.41
WU + 0.500 mg/ml	6.97	58.76
WU + 1.00 mg/ml	7.21	48.24

### **SEM**

Figure 4.5 shows the SEM micrographs of CaOx crystals precipitated from the urine of black and white subjects before and after addition of lime solutions. The crystals were all viewed at 4000X magnification. Figures 4.5.1-4.5.4 represent the deposited crystals of black subjects while Figures 4.5.5-4.5.8 represent those of the white subjects.

Crystal numbers increased with increasing lime concentrations in black subjects while decreased in white subjects. On the other hand, CaOx crystal sizes tended to remain unchanged in black subjects but appeared to be bigger in white subjects after the addition of lime solutions, relative to the control. The number of aggregated crystals in the urines of white subjects decreased with increasing lime concentrations whereas aggregated crystals were not observed in black subjects. A mixture of COM and COD crystals were observed in the control samples of black subjects, but COM crystals were precipitated as lime was added. On the other hand, COD crystals were observed in white subjects before and after the addition of lime concentrations.



#### *CaOx crystal nucleation and aggregation*

Table 4.5 shows that the mean % of inhibition of CaOx nucleation showed a general tendency to decrease in both groups thereby indicating a tendency towards promotion of nucleation. It is also noted that the values for white subjects were generally higher, thereby suggesting that promotion of nucleation is greater in the black group.

The mean % inhibition of CaOx aggregation in the urine of black subjects increased consistently as the lime concentrations increased, achieving statistical significance at the two highest concentrations. On the other hand, no obvious trend was apparent in the urine of white subjects. Typical plots of each subject are shown in Appendix 3.

**Table 4.5:** Mean percentage inhibition (% In) of CaOx crystal nucleation and aggregation as a function of lime concentrations in the urine of black and white subjects (SE)

<i>Samples</i>	<i>% In of Nucleation</i>	<i>P- values</i>	<i>% In of Aggregation</i>	<i>P-values</i>
BU	67.2 (7.258)		62.6 (9.197)	
BU + 0.125 mg/ml	60.4 (9.448)	0.345	69.8 (10.23)	0.323
BU + 0.500 mg/ml	55.2 (9.851)	0.108	77.6 (9.271)	0.045*
BU + 1.00 mg/ml	57.8 (7.319)	0.200	81.3 (7.348)	0.014*
WU	82.9 (7.258)		77.9 (9.197)	
WU + 0.125 mg/ml	60.2 (9.448)	0.005*	69.8 (10.23)	0.260
WU + 0.500 mg/ml	70.4 (9.851)	0.100	72.0 (9.271)	0.413
WU + 1.00 mg/ml	73.9 (7.319)	0.231	82.4 (7.348)	0.533

\*Significance at  $p < 0.05$

#### *CaOx crystal growth*

Table 4.6 shows the mean % inhibition of CaOx growth in both groups. It is noted that there is a general tendency for this property to increase in both groups with increasing lime concentrations. Plots of each subject of these experiments are shown in Appendix 3.

**Table 4.6:** Mean percentage inhibition (% In) of CaOx crystal growth as a function of lime concentrations in the urine of black and white subjects (SE)

<i>Samples</i>	<i>% In of Growth</i>	<i>P-values</i>
BU	-7.0 (5.06)	
BU + 0.125 mg/ml	1.4 (6.17)	0.208
BU + 0.500 mg/ml	3.5 (7.17)	0.005*
BU + 1.00 mg/ml	2.1 (5.89)	0.176
WU	-11.2 (5.06)	
WU + 0.125 mg/ml	-6.9 (6.17)	0.516
WU + 0.500 mg/ml	-1.5 (7.17)	0.152
WU + 1.00 mg/ml	5.7 (5.89)	0.016*

\*Significance at  $p < 0.05$

**Free calcium ( $Ca^{2+}$ ) and BRI**

Mean free  $Ca^{2+}$  concentrations and BRI values for both groups are given in Table 4.7. It is noted that mean free  $Ca^{2+}$  in black subjects showed no difference after dosing with different lime solutions, while for white subjects a significant decrease occurred after dosing with 1.00 mg/ml concentration. Mean BRI values for both groups decreased with increasing concentrations of lime powder. It is also noted that black subjects have lower BRI values than their white counterparts.

**Table 4.7:** The mean percentage of free  $Ca^{2+}$  concentration and BRI values in the absence and presence of lime solutions for black and white subjects (SE)

<i>Samples</i>	<i>Free <math>Ca^{2+}</math></i>	<i>P-values</i>	<i>BRI</i>	<i>P-values</i>
BU	0.306 (0.072)		0.826 (0.417)	
BU + 0.125 mg/ml	0.303 (0.061)	0.883	0.951 (0.346)	0.639
BU + 0.500 mg/ml	0.298 (0.054)	0.644	0.874 (0.361)	0.855
BU + 1.00 mg/ml	0.298 (0.050)	0.644	0.814 (0.336)	0.965
WU	0.444 (0.072)		1.940 (0.414)	
WU + 0.125 mg/ml	0.430 (0.061)	0.421	1.876 (0.346)	0.809
WU + 0.500 mg/ml	0.417 (0.054)	0.129	1.844 (0.361)	0.719
WU + 1.00 mg/ml	0.401 (0.050)	0.017*	1.740 (0.336)	0.451

\*Significance at  $p < 0.05$

## 4.3.4 Summary

The results of various crystallization experiments are summarised and briefly interpreted in Table 4.8.

**Table 4.8:** A summary of results and interpretation showing the effect of lime concentrations (0.125 mg/ml-1.00 mg/ml) in samples from black and white subjects

<i>Variables</i>	<i>Blacks</i>	<i>Whites</i>	<i>Interpretation: Effect of lime</i>
MSL	no change	no change	does not affect initiation of nucleation in each group
PSD size	↓	↓	inhibits growth in both groups
volume	no change	↓	inhibits growth or nucleation or aggregation in whites
SEM size	no change	↑	promotes growth in whites
number	↑	↓	promotes nucleation in blacks, inhibits nucleation in whites
% inhibition of crystal nucleation	↓	↓	promotes nucleation in both groups
% inhibition of crystal aggregation	↑	↑	inhibits aggregation in both groups
% inhibition of crystal growth	↑	↑	inhibits growth in both groups
Free Ca <sup>2+</sup>	↓	↓	binds Ca <sup>2+</sup> in both groups
BRI	↓	↓	reduces risk in both groups

#### 4.4 DISCUSSION

Interesting results have been observed in the urine from both groups in this study. CaOx MSL was demonstrated to be higher in black subjects indicating that induction of crystallization is less easily achieved in this group. This is commensurate with the rarity of stones in blacks.

Lime did not affect the MSL in either group. This is not unusual as other inhibitory substances such as citrate (Ryall et al 1985), urinary glycosaminoglycans (GAGs) e.g. heparin sulphate (Suzuki and Ryall 1996), and proteins e.g. Tamm-Horsfall mucoprotein and prothrombin fragment 1 (Grover et al. 1994; Grover and Ryall 2002) have also been reported as having no effect on CaOx metastable limit.

Table 4.8 shows that lime induced the same effects in both groups except in the PSD (volume) determinations and in the SEM investigations. Regarding the effects common to both groups, it is noted that lime promoted CaOx crystal nucleation (nucleation assay), inhibited growth (growth assay) and inhibited aggregation (aggregation assay).

Robertson and Scurr (1986) noticed that citrate was able to promote nucleation. The present results show that the addition of lime solutions to the urine of both groups promoted nucleation of CaOx crystals. The authors also showed that citrate was able to reduce the crystal growth. Hennequin et al. (1993) with another model of crystallization confirmed that citrate showed 50 % inhibition of crystal growth.

Kok et al. (1986, 1988) in their studies about citrate on *in vitro* crystallization model, showed that citrate had a strong activity against crystal aggregation. Another *in vitro* study by Kulaksizoğlu et al. (2007) on lemon and orange juices showed that lemon which is also rich in citrate was found to inhibit the rate of crystal aggregation. On that note, the present results showed that lime had also the capability of inhibiting CaOx crystal growth and aggregation for both population groups as seen in Table 4.8. This is promising and supports what has already been reported in the literature.

These are all very favourable outcomes in the context of urolithiasis as each represents a reduced risk of stone formation. The PSD (size) results support the aforementioned conclusion that lime inhibits CaOx crystal growth in the urine of both groups. In addition pH was not affected by lime solutions. However, this is in conflict with the SEM results which suggest that growth is promoted in whites.

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Since inhibition of growth is also indicated by the PSD (volume) results in whites, it is likely that this protective mechanism is indeed occurring.

The only other result which contradicts the assay interpretations is the SEM (number) observation of a decrease in whites which suggest inhibition of nucleation. Since the assay and the SEM studies must be regarded as being of equal credibility, it is not possible to draw a firm conclusion about the effect of lime on nucleation in the urine of whites. However, despite this uncertainty, the two remaining variables, (free  $\text{Ca}^{2+}$  and BRI) both demonstrate empirically that lime lowers the risk of CaOx crystallization in the urines of black and white subjects.

Another distinction between the urinary responses in black and white subjects is that the former precipitated COM crystals while the latter precipitated COD crystals. The suggested reason for the COD precipitated crystals in white subjects is that they have higher urinary calcium concentrations which as a result favour the formation of these crystals (Webber et al. 2002). Although urinary calcium was not measured in this study, the high intake of calcium in this group as shown in Table 4.3 might suggest that their urinary calcium was also high as compared to blacks. The formation of COD crystals is of great importance because they show a lower binding affinity for epithelial cell surfaces than COM crystals, thus inhibiting the critical step in the formation of kidney stones (Wesson et al. 1998). These results showed that there is a difference between the two South African population groups which confirms what has already been reported in the literature. They also show that similar results to that obtained in the AU study were also observed in this study, once again suggesting that lime has favourable effects in the context of CaOx stone formation

When combined with the favourable reduction in the other risk factors described above, a clinical trial involving the ingestion of lime is strongly justified. This is described in the following chapter.

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**CHAPTER FIVE**  
**CLINICAL TRIAL TO INVESTIGATE**  
**THE EFFECT OF THE INGESTION OF**  
**LIME POWDER SOLUTION ON**  
**CALCIUM OXALATE**  
**CRYSTALLIZATION IN REAL URINE**

## 5.1 INTRODUCTION

Citrate is an important inhibitory substance as it forms soluble complexes with calcium in urine. It reduces the saturation of CaOx and CaP and inhibits crystallization (Pak 1987). As mentioned in Chapter 1, citrus fruits and juices (lemon, orange, grapefruits, and lime) are a dietary source of citrate and therefore could represent an alternative to pharmacological citrate therapy.

Several studies have investigated the impact of citrus juices on urinary parameters in kidney stone patients and some in healthy subjects. Wabner and Pak (1993) showed that orange juice caused an increase in urinary citrate and oxalate levels while it decreased calcium excretion. More studies on lemon juice (Seltzer et al. 1996; Kang et al. 2007; Penniston et al. 2007; Aras et al. 2008) showed an increased urinary citrate excretion. They also showed increased urinary pH and decreased calcium levels after the ingestion of lemon juice. Goldfarb and Asplin (2001) reported that citrate and oxalate levels were increased in healthy subjects after the intake of grapefruit juice for 7 days. The effect of lime juice on urinary pH investigated by Mazdak et al. (2006) in healthy subjects showed that the juice increased the urinary pH, while in another study by Tosukhowong et al. (2008) on nephrolithiasis patients, lime increased urinary citrate, pH and potassium.

Having established that solutions of lime favourably alter the urinary risk factors for CaOx stone formation *in vitro*, it makes sound scientific sense to investigate its effect *in vivo*. This section focuses on whether lime powder could be a therapeutic agent for nephrolithiasis as suggested by the previously described *in vitro* studies and also compares the handling of this citrus fruit by subjects from South Africa's two population groups.

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## 5.2 MATERIALS AND METHODS

### 5.2.1 Study design

5 black and 5 white age-matched South African males (ages 20-28 years), were recruited from the undergraduate and postgraduate cohorts of the University of Cape Town. However, one of the 5 white subjects was subsequently excluded because of his extremely high urinary creatinine value. None of the subjects in either group had metabolic illness or any clinical problems. They were instructed to stop taking any supplements three days before commencement of the study. None of the subjects had a history of kidney stones.

For each subject, brief information about their social and medical history was collected as explained in Chapter 4.2 and an instruction sheet on how to collect the 24-hour urine and to take lime powder, was provided (Appendix 4). Those who agreed to participate in the study after a verbal and written explanation were given a letter of consent to sign (Appendix 4).

Each subject was asked to collect a 24-hour baseline urine before the ingestion of lime powder. They were given seven sachets of lime powder (5g each) to drink with warm water every day. On the 7<sup>th</sup> day, subjects were again asked to collect a 24-hour urine.

As with the *in vitro* study, subjects were on a free diet but were advised to avoid certain foods (Table 4.2). They were instructed to control their diet by eating the same food on the baseline day and on day 7, as implemented by Karagülle et al. (2007). A 24-hour dietary recall questionnaire was used to record food and drinks ingested at baseline and on day 7 as explained in Chapter 4.2.

### **5.2.2 Experimental methods**

#### ***Urine collection and analysis***

24-hour urine samples were collected in plastic bottles without preservative from the recruited subjects at baseline and on day 7. Each sample was tested for haematuria and nitrite using urinalysis test strips and those that tested positive for either were discarded. All urine samples were analysed for pH, sodium, potassium, calcium, oxalate, urate, citrate, chloride, magnesium, phosphate and creatinine as described in Chapter 2.2.3 (page 52).

#### ***Crystallization experiments***

The CaOx MSL, PSD, crystal nucleation and aggregation inhibition, and CaOx crystal growth assay were determined in the baseline samples and in samples after the ingestion of lime powder. The techniques have been described in Chapter 2.

#### ***Free calcium ion concentrations and BRI***

These parameters were determined as described in Chapter 2.2.5 (page 60). 100 ml of each urine sample collected before and after the ingestion of lime powder was used to measure free calcium ( $\text{Ca}^{2+}$ ) and BRI.

#### ***Statistical analysis***

Statistical analyses were conducted as described in Chapter 2.2.8 (page 61).

## **5.3 RESULTS**

### **5.3.1 Social and medical history**

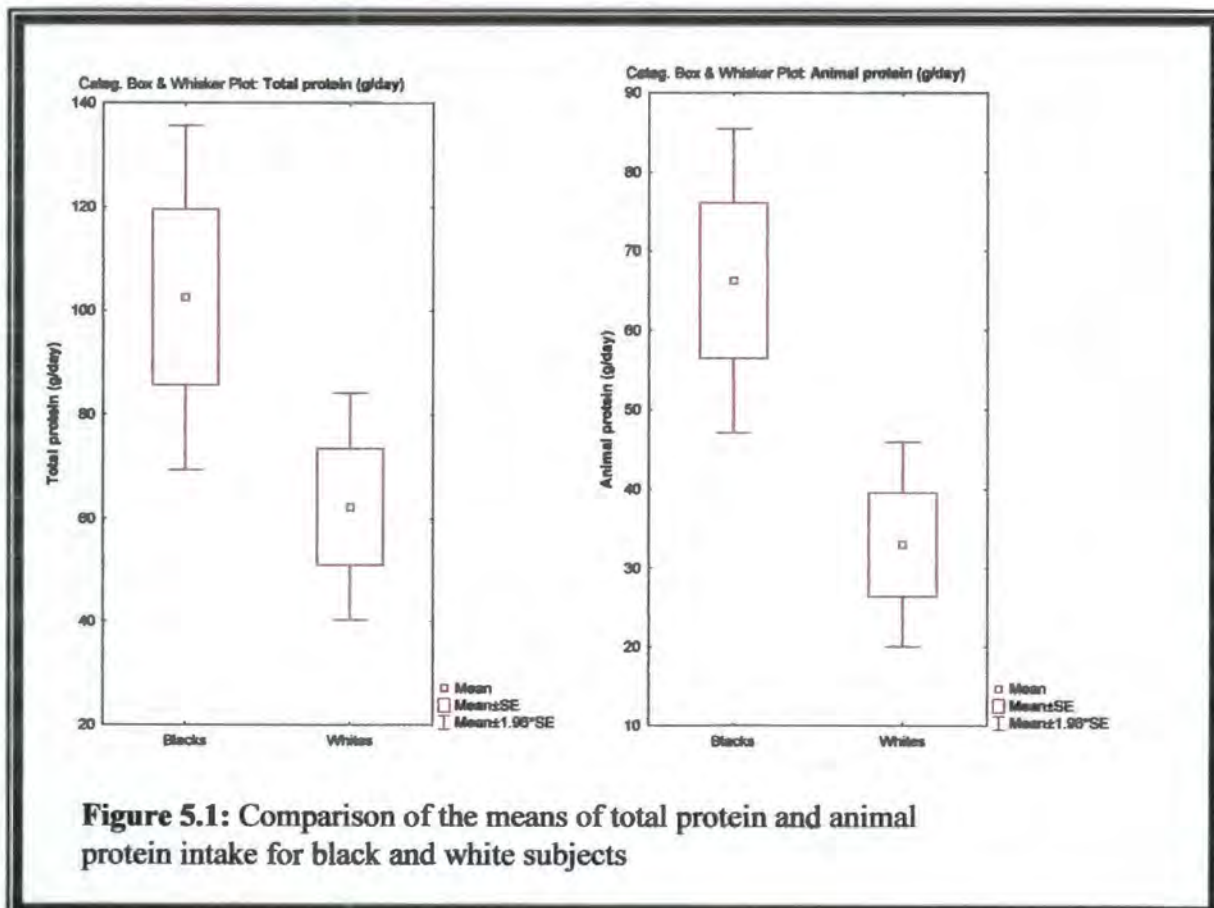
As in the *in vitro* study, it was found that black subjects had higher weight hence high BMI as shown in Table 5.1. They were not on any specific diet and most of them were not exercising but were exposed to alcohol. On the other hand, two white subjects were on supplements (but were asked to stop taking them 3 days before the commencement of the study as explained in Chapter 2.2 on page 52). White subjects were exposed to alcohol and they were engaged in different kinds of exercises. None of the subjects had experienced recent illness or were taking any medication prior the study.

### **5.3.2 Dietary analysis**

The mean intake of nutrients for the two groups was determined from the completed dietary questionnaires. The values are given in Table 5.1. Because the subjects were asked to ingest the same food on both days, the average intake of nutrients was used for this study. Subjects who were non compliant with their diet were eliminated from the study. This was observed from the 24-hour dietary recall questionnaire which was used to record food and drinks ingested. Total protein and animal protein were the only nutrients which were significantly different between the groups; consumption in both cases being higher for the black subjects. The mean with standard error of both total protein and animal protein is illustrated graphically in Figure 5.1. The daily nutrient intake of each subject is given in Appendix 5.

**Table 5.1:** Mean daily intake of nutrients for black and white subjects

<i>Variables</i>	<i>Blacks (n =5)</i>	<i>Whites (n =4)</i>	<i>P-values</i>
Moisture	1104 (304.3)	1413 (304.3)	0.155
Total Protein (g/day)	110.2 (13.46)	54.16 (13.46)	0.036*
Animal Protein (g/day)	66.28 (8.352)	29.00 (8.352)	0.023*
Total fat (g/day)	60.52 (12.25)	48.04 (12.25)	0.677
Carbohydrate (g/day)	280.8 (44.80)	216.3 (44.80)	0.382
Fibre (g/day)	33.78 (6.074)	18.00 (6.074)	0.175
Added sugar (g/day)	58.44 (17.30)	58.70 (17.30)	0.913
Oxalate (mg/day)	18.40 (12.76)	49.60 (12.76)	0.274
Ca (mg/day)	529.0 (106.8)	555.0 (106.8)	0.683
Mg (mg/day)	339.0 (64.49)	254.0 (64.49)	0.620
Phosphate (mg/day)	1320 (178.6)	897 (178.6)	0.289
K (mg/day)	2786 (410.5)	2113 (410.5)	0.410
Na (mg/day)	2196 (347.7)	1366 (347.7)	0.179
Vitamin A (RE/day)	679.0 (271.3)	1048 (271.3)	0.170
Vitamin B <sub>6</sub> (mg/day)	2.214 (0.343)	2.000 (0.343)	0.290
Vitamin C (mg/day)	193.6 (67.24)	40.23 (67.24)	0.130
Vitamin D (µg/day)	4.428 (1.268)	2.955 (1.268)	0.363
BMI	28.42 (2.266)	24.7 (2.266)	0.243

\*Significance at  $p < 0.05$ 

### 5.3.3 Urine composition

Urinary parameters and computed risk indices for both race groups prior the administration of lime powder are given in Table 5.2. It is noted that the urinary magnesium is significantly lower in blacks (1.80 vs 3.86 mmol/24h,  $p = 0.001$ ) and also the Tiselius risk index (TRI) (computed from urinary calcium, magnesium, citrate, oxalate and creatinine) is significantly lower in this group compared to whites (203 vs 304,  $p = 0.0102$ ).

Comparison of parameters before and after the ingestion of lime powder within each group is also shown in the same table (Table 5.2). The table shows that the only urinary variables which changed significantly were magnesium and phosphate excretions which increased in black subjects (1.80 vs 2.75 mmol/24h,  $p = 0.0001$  and 20.9 vs 24.6 mmol/24h,  $p = 0.023$  respectively). On the other hand, a decrease in urinary oxalate after 7 days of lime powder ingestion (0.313 vs 0.205 mmol/24h,  $p = 0.023$ ) was observed in whites. TRI was also decreased significantly in this group (304 vs 187,  $p = 0.019$ ). The urine composition and computed risk indices of each subject are given in Appendix 6.

**Table 5.2:** Comparison of mean urinary parameters (SE) before and after the ingestion of lime powder in both race groups

<i>Variables</i>	<i>Blacks (n=5)</i>			<i>Whites (n=4)</i>			<i>Blacks vs Whites</i>
	<i>Baseline</i>	<i>Day 7</i>	<i>P values</i>	<i>Baseline</i>	<i>Day 7</i>	<i>P-values</i>	<i>Baseline P-values</i>
pH	6.28 (0.116)	6.04 (0.116)	0.223	5.71 (0.071)	6.02 (0.071)	0.346	0.112
Volume (mL/24h)	1475 (215.6)	1878 (215.6)	0.257	1805 (89.3)	1500 (89.3)	0.438	0.514
Citrate (mmol/24h)	2.04 (0.338)	2.96 (0.338)	0.125	2.64 (0.786)	5.56 (0.786)	0.0894	0.269
Oxalate (mmol/24h)	0.222 (0.042)	0.294 (0.042)	0.294	0.313 (0.0306)	0.205 (0.0306)	0.023*	0.118
Calcium (mmol/24h)	2.82 (0.055)	2.98 (0.055)	0.114	4.44 (0.271)	4.04 (0.271)	0.736	0.116
Magnesium (mmol/24h)	1.80 (0.055)	2.75 (0.055)	0.0001*	3.86 (0.186)	4.16 (0.186)	0.598	0.001*
Sodium (mmol/24h)	149 (26.2)	144 (26.2)	0.913	126 (7.07)	129 (7.07)	0.867	0.765
Potassium (mmol/24h)	50.9 (6.53)	67.0 (6.53)	0.157	57.6 (7.19)	72.6 (7.17)	0.249	0.612
Urate (mmol/24h)	2.98 (0.252)	3.36 (0.252)	0.347	2.88 (0.423)	3.38 (0.423)	0.663	0.904
Creatinine (mol/24h)	14.2 (0.782)	14.7 (0.782)	0.663	15.5 (2.31)	16.4 (2.31)	0.836	0.557
Phosphate (mmol/24h)	20.9 (0.714)	24.6 (0.714)	0.023*	26.2 (3.77)	29.8 (3.77)	0.619	0.304
Chloride (mmol/24h)	145 (34.4)	153 (34.4)	0.880	133 (25.9)	143 (25.9)	0.801	0.840
RS COM	6.66 (0.269)	7.24 (0.269)	0.199	7.61 (0.0792)	7.26 (0.0792)	0.374	0.141
RS COD	2.65 (0.136)	3.08 (0.136)	0.088	3.24 (0.0336)	3.09 (0.0336)	0.381	0.143
RS Brushite	3.87E-09 (0.00)	3.81E-09 (0.00)	0.979	2.66E-09 (0.00)	2.08E-09 (0.00)	0.679	0.678
RS Uric Acid	3.62 (0.704)	4.82 (0.704)	0.296	5.93 (0.284)	4.99 (0.284)	0.517	0.188
Tiselius risk index	203 (26.9)	243 (26.9)	0.346	304 (29.4)	187 (29.4)	0.019*	0.0102*

\*Significance at  $p < 0.05$

### 5.3.4 Crystallization Parameters

Results of the crystallization parameters are shown in Table 5.3. It is apparent that there were no statistically significant differences between the groups for any of the crystallization parameters at baseline. However, it is noted that free  $\text{Ca}^{2+}$  and BRI values tend to be lower in blacks while MSL values tend to be higher.

On the other hand, the same table shows a comparison of the crystallization variables before and after ingestion of lime powder. A significant increase of the inhibition of CaOx crystal aggregation in black subjects was observed after day 7 (72.9 vs 78.1,  $p = 0.031$ ). No other significant differences occurred, although there was an increase in the MSL for whites which is approaching significance. The MSL data and plots of each urine sample are shown in Appendix 1 while the plots for nucleation, aggregation and growth assays for each subject are shown in Appendix 3.

**Table 5.3:** Comparison of mean variables (SE) before and after the ingestion of lime powder in both race groups

Variables	Blacks (n=5)			Whites (n=4)			Blacks vs Whites
	Baseline	Day 7	P values	Baseline	Day 7	P-values	Baseline P-values
% In of Nucleation	68.9 (2.30)	70.7 (2.30)	0.607	71.9 (4.35)	67.6 (4.35)	0.526	0.794
% In of Aggregation	72.9 (1.07)	78.1 (1.07)	0.031*	71.1 (6.49)	69.1 (6.49)	0.844	0.763
% In of Growth	28.1 (0.130)	-2.572 (0.130)	0.171	0.635 (0.793)	10.05 (0.793)	0.381	0.463
Free $\text{Ca}^{2+}$	0.181 (0.062)	0.290 (0.062)	0.280	0.684 (0.126)	0.415 (0.126)	0.229	0.176
BRI	0.592 (0.189)	0.563 (0.189)	0.921	2.75 (0.820)	1.00 (0.820)	0.228	0.213
MSL (mmol/l)	78.0 (10.9)	87.00 (10.9)	0.591	48.7 (7.95)	82.5 (7.95)	0.057	0.282

\*Significance at  $p < 0.05$

**CaOx PSD**

The PSD of both groups are illustrated in Figure 5.2. The graphs show that the particle sizes for both groups did not change statistically (4.32 vs 5.54  $\mu\text{m}$  for blacks;  $p = 0.494$  and 6.014 vs 6.912  $\mu\text{m}$  for white;  $p = 0.959$ ). Particle volumes for black subjects did not change (88.8 vs 91.3  $\times 10^3 \mu\text{m}^3/500 \mu\text{l}$ ) while for white subjects, the particle volume increased (102.8 vs 119.3  $\times 10^3 \mu\text{m}^3/500 \mu\text{l}$ ). The PSD plots of each subject are shown in Appendix 2.

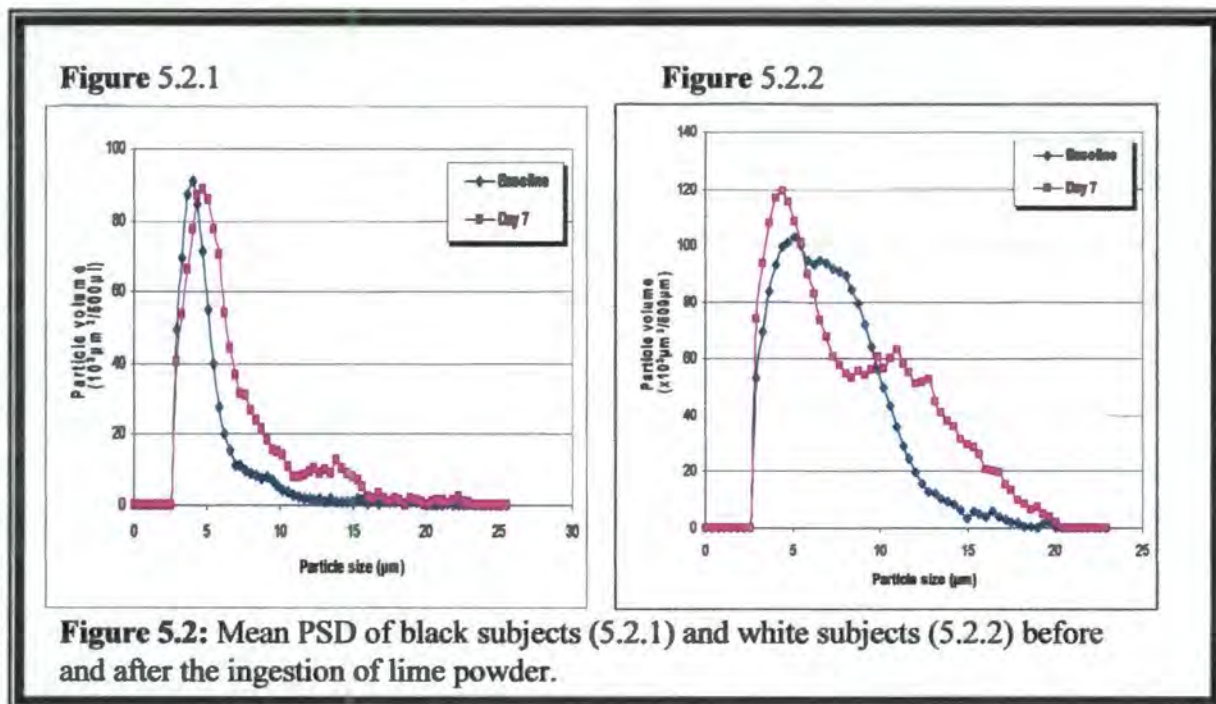
**SEM**

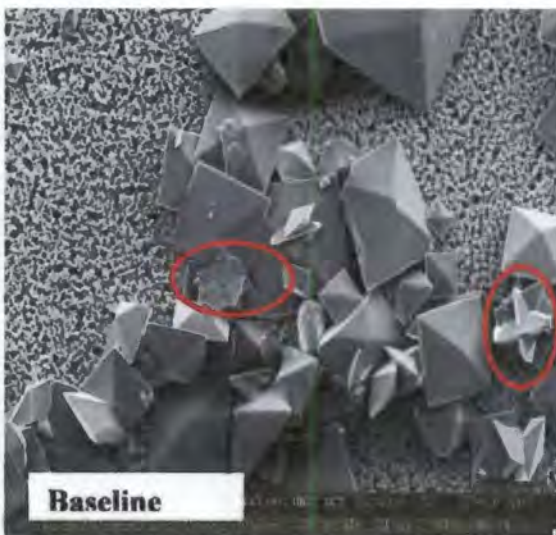
Figure 5.3 shows scanning electron micrographs of crystals deposited after the induction of CaOx crystallization in urines from both black and white subjects. The crystals were all viewed at 4000X magnification.

Figure 5.3.1 shows that only COM crystals were deposited at baseline in blacks while Figure 5.3.2 shows that crystal numbers decreased after 7 days of ingesting the lime powder.

On the other hand, Figure 5.3.3 represents the micrographs of urine crystals from white subjects at baseline. This shows relatively large COD crystals, some of which are aggregated (circled in red), whereas Figure 5.3.4 shows that fewer COD crystals of smaller sizes were precipitated after 7 days of lime ingestion. No aggregated crystals were observed as compared to the baseline micrograph.

**Figure 5.3.1****Figure 5.3.2**

**Figure 5.3:** Scanning electron micrographs of CaOx crystals induced from the urine of black subjects before (baseline) and after the ingestion of lime powder (day 7)

**Figure 5.3.3****Figure 5.3.4**

**Figure 5.3:** Scanning electron micrographs of CaOx crystals induced from the urine of white subjects before (baseline) and after the ingestion of lime powder (day 7). The circled crystals depict the aggregated ones.

## 5.3.5 Summary

The results of various urine composition and crystallization experiments are summarised and briefly interpreted in Table 5.4.

**Table 5.4:** Summary of the urine composition and crystallization results after the ingestion of lime powder for 7 days.

<i>Variables</i>	<i>Blacks</i>	<i>Whites</i>	<i>Interpretation: Effect of lime</i>
Urinary risk factors	magnesium ↑ phosphate ↑	oxalate ↓ TRI ↓	reduces the risk in whites promotes risk in blacks due to increased urinary phosphate
MSL	no change	no change	no effect on initiation of nucleation
PSD size	no change	no change	
volume	no change	↑	promotes nucleation, growth or aggregation in whites
SEM size	no change	↓	inhibits growth in whites
number	↓	↓	inhibits nucleation in both groups
aggregation	no change	↓	inhibits aggregation in whites
% Inhibition of crystal nucleation	↑	↓	inhibits nucleation in blacks promotes nucleation in whites
% Inhibition of crystal aggregation	↑	↓	inhibits aggregation in blacks promotes aggregation in whites
% Inhibition of crystal growth	↓	↑	promotes growth in blacks inhibits growth in whites
Free Ca <sup>2+</sup>	↑	↓	binds Ca <sup>2+</sup> in whites and not in blacks
BRI	↓	↓	reduces risk in both groups

#### 5.4 DISCUSSION

Table 5.4 shows the summary of the effect of lime powder in black and white groups. The table shows that lime powder acted favourably on two urinary parameters in white subjects. Oxalate is a urinary parameter which is regarded as being a highly important factor in CaOx kidney stones formation (Borsatti 1991). Because there is much less oxalate than calcium in the urine, it means that small increases in oxalate concentration has a far greater impact than changes in calcium concentration. Thus, the decrease in urinary oxalate in the white subjects after the ingestion of lime powder is of great importance. In the previous study by Tosukhowong et al. (2008) involving urolithiasis patients (wherein patients were given lime powder) for a period of 3 months, they did not show a significant decrease in oxalate excretion.

The TRI in the present study also decreased favourably in this group after the ingestion of lime powder.

Crystallization experiments indicate that lime promotes nucleation (PSD vol, nucleation assay) in whites. However, this is contradicted by SEM observations which suggest inhibition of this process. This is inconclusive. However, there is a strong empirical evidence to suggest that lime inhibits CaOx growth in this group (SEM, growth assay). Aggregation is promoted (PSD vol, aggregation assay). This is however contradicted by SEM. Therefore the results in whites are inconclusive with respect to nucleation and aggregation, but supportive of growth inhibition. Also, very importantly, free  $\text{Ca}^{2+}$  and BRI decreased (both are influential risk factors). Therefore the overall conclusion is that lime had favourable effects for reducing the risk of CaOx kidney stones in this group.

In black subjects, a favourable increase of urinary magnesium was accompanied by an unfavourable increase in urinary phosphate (which is a risk factor for CaP stones) after the ingestion of lime powder. Magnesium is one of the micromolecular urinary inhibitors (Moe 2006). It has been shown to decrease the crystallization of CaOx by complexing with oxalate ions to form soluble magnesium oxalate, meaning that magnesium can reduce the amount of free oxalate ions in the urine (Desmars and Tawashi 1793; Hallson et al. 1982; Danielson 1985). However, this is contradicting because there was no significant decrease of oxalate excretion in this group after the ingestion of lime powder.

Crystallization experiments showed that lime inhibits nucleation (SEM, nucleation assay) in this group. Although it has been previously stated that promotion of nucleation is favourable, this mechanism is not as important as growth and aggregation. Therefore, the increase in nucleation is not crucial, but must nevertheless be considered as unfavourable. Lime showed an unfavourable promotion of crystal growth (growth assay) but a favourable inhibition of aggregation (aggregation assay). It also showed an unfavourable increase in free  $\text{Ca}^{2+}$  but a favourable decrease in BRI. These results indicate that lime powder had an inconclusive effect on reducing the urinary risk factors in black subjects.

SEM observations revealed that COD crystals were precipitated in the baseline samples of white subjects, while black subjects deposited only COM crystals. The precipitation of COD crystals in white subjects is in agreement with the higher urinary calcium (albeit insignificant) excretion, which favours the formation of these crystals (Webber et al. 2002). As mentioned in the previous paragraphs, lime powder affected other crystallization parameters differently in these two groups. Therefore, the present study has provided further evidence of different renal or gastrointestinal handling mechanisms in these two groups, in response to ingestion of lithogenic and antilithogenic challenges.

Regarding its potential as a therapeutic agent, the effect of lime in black subjects is inconclusive. However, several favourable results were observed in white subjects which suggest that it may indeed have such a potential.

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***CHAPTER SIX***  
***GENERAL DISCUSSION AND***  
***CONCLUDING REMARKS***

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## 6.1 DISCUSSION AND CONCLUDING REMARKS

The effect of citrus fruit (lime) on CaOx crystallization in the urine of South Africa's population groups has not been previously investigated. Therefore the objective of this dissertation was to measure the relative inhibitory activity of lime, (in the form of a powder), in artificial and real urine and to investigate its effect in black and white subjects.

As much as the artificial urine is different from the more complex real urine, the *in vitro* study of the real urine (Chapter 4) showed that the effect of lime solutions in the two media is similar. The CaOx MSL of both AU and real urine (Chapters 3 and 4) as well as that shown in the trial study (Chapter 5) showed that it is independent of the concentration of the lime solutions. The MSL of AU is different to that of real urine because the two media are different. AU is a simple inorganic solution while real urine is very complex with many micro/macro molecules present. The results of the present study show that lime does not affect the MSL in either of these solutions. This is not surprising because other inhibitory substances like citrate (Ryall et al. 1985), urinary glycosaminoglycans (GAGs) e.g. heparin sulphate (Suzuki and Ryall 1996), and proteins e.g. Tamm-Horsfall mucoprotein and prothrombin fragment 1 (Grover et al. 1994; Grover and Ryall 2002) also do not have an effect on the CaOx MSL.

Similar results in the AU and real urine studies (Chapters 3 and 4) were observed for 3 crystallization mechanisms. These included promotion of nucleation and inhibition of both growth and aggregation after the addition of lime solutions in these two media. However, lime affected these parameters differently in the trial study (Chapter 5). In addition, lime treatment did not result in the same volume reduction as was the case when lime was added *in vitro*. Differences in urine composition might explain that. The reason might be the fact that there is a difference between dosing urine directly with lime solutions and ingestion thereof. The latter takes into account many metabolic processes that take place within the gastrointestinal tract following ingestion.

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Previous researchers have reported that South African blacks have a decreased intake of calcium and protein, both of which are regarded as risk factors for stone formation. In contrast to these reports, black subjects have in the present project showed a higher intake of total protein compared to whites due to a high intake of animal protein (Chapter 5). The same results were not observed in Chapter 4. It is further noted that a significantly low intake of calcium in black subjects observed in Chapter 4 was not observed in the trial study (Chapter 5). Although it is surprising that these dietary intakes differed in these two studies as they were expected to give similar results, the reason might be due to the small number of subjects who participated, thereby diminishing the statistical power of the studies.

An interesting feature which occurred in the real urine studies (Chapters 4 and 5) regarding the differences between urine of black and white subjects was that the urine of white subjects showed predominantly COD crystal morphology while black subjects demonstrated COM morphology. This observation is not surprising since COD forms at higher calcium concentrations and as mentioned before, white subjects have higher calcium intake and therefore higher urinary calcium excretion compared to blacks. In addition, the precipitation of COD crystals in this group is important because these crystals show a lower binding affinity for epithelial cell surfaces than COM crystals, thus inhibiting the crucial step in the formation of kidney stones (Wesson et al. 1998).

The BRI values which were higher in whites than in blacks in both studies and the lower CaOx risk index (TRI) in blacks also showed yet another difference between these groups and these observations are consistent with blacks' lower stone incidence.

Differences between the black and white responses in the present study support the general hypothesis which has been reported by researchers from the Kidney Stone Research Laboratory (University of Cape Town), (Lewandowski et al. 2001; Rodgers and Lewandowski 2002; Lewandowski et al. 2005), namely that the black and white population groups in South Africa have different renal handling when lithogenic and anti-lithogenic (in the present study, anti-lithogenic) nutrients are administered.

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Surprisingly, lime powder failed to significantly increase the urinary citrate excretion in both groups. Other non-parametric statistical tests also failed to reveal a significant increase. This was not expected because lime has a high content of citrate and the increase in urinary citrate excretion has been observed with other citrus studies (Hönow et al. 2003; Kang et al. 2007; Penniston et al. 2007; Aras et al. 2008; Tosukhowong et al. 2008). Contrarily, a citrus (lemon) study by Koff et al (2007) showed that lemon juice did not improve the urinary citrate. However, the failure of lime powder to increase urinary citrate in the present study may be due to natural difference in absorbing or handling citrate compared to those of previous studies, albeit that there is no evidence to support this notion. The other possible reason might be that the effect of lime requires more than 7 days of regular ingestion to become apparent.

Nevertheless, although lime powder failed to increase urinary citrate in either group, it however favourably decreased various urinary CaOx risk factors (urinary oxalate, TRI, free Ca<sup>2+</sup> and BRI) in the white group. This was not observed in the black group.

The overall results of the various studies presented in this dissertation lead to the conclusion that lime powder may be regarded as a potential therapeutic agent for reducing the risk of CaOx kidney stones in the white group. The results also support the evidence that there is a difference between black and white subjects in their handling of lithogenic and antilithogenic dietary challenges.

Rigorous controlled trials in healthy subjects and in kidney stone patients need to be conducted in future studies to unambiguously confirm these findings.

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