In vitro studies on the effect of fucoidan on calcium oxalate crystallization
2.1. INTRODUCTION

The natural progression of the urinary chemistry leading to stone development is urine saturation, supersaturation, crystal nucleation, aggregation, growth, retention and stone development. Compounds which are capable of interfering with nucleation and aggregation phases can be effective modulators of crystallization process. In urolithiasis research, a wide variety of in vitro crystallization models are in use for examining the effects of numerous additives. In vitro crystallization studies help in the identification of critical molecules which could modulate crystallization process and hence, serve as essential tools to analyze the effect of a drug on crystallization process.

In recent years, substantial evidence supports the inhibitory role of GAGs in urolithiasis (Cao et al., 1997a). Considerable heterogeneity does exist among the GAGs regarding their effect on crystallization process. Several in vitro models have demonstrated the inhibitory effect of urinary GAGs on calcium oxalate stone formation. Ryall et al. (1981) reported that heparin and chondroitin sulphate were responsible for the major portion of the inhibitory effect on aggregation of calcium oxalate crystals. The inhibitory effect of the chondroitin-4-sulphate and chondroitin-6-sulphate was also confirmed by Bowyer et al. (1979). However, Roberts and Resnick (1986) presumed that GAGs are intimately related to the stone development and suggested that GAGs might even induce the formation of stones in the urinary tract (Michelacci et al., 1992). Since the role of GAGs on crystallization process is confounding, it becomes essential to study the effect of fucoidan, the naturally occurring GAGs from F. vesiculosus on the crystallization process. Hence, the forthcoming section was oriented to probe the effect of fucoidan on crystallization events using in vitro assay
systems. The effect of fucoidan was compared with a GAG and an effective inhibitor of crystallization, heparan sulphate.

2.2. MATERIALS AND METHODS

Fucoidan from *F. vesiculosus*, heparan sulphate were obtained from Sigma Chemicals, St. Louis, MO, USA. All other chemicals and solvents used were of analytical grade.

2.2.1. Spectrophotometric crystallization assay

This assay was done according to the method of Hess *et al.* (1995). To 1 ml of potassium oxalate solution taken in the quartz cuvette, 1 ml of calcium chloride solution was added, so that the final concentration of the incubation mixture was 4.25 mM for calcium and 0.75 mM for oxalate. All the solutions were prepared in deionized water containing 200 mM NaCl and 10 mM sodium acetate (pH 5.7). Automated time course measurements of optical density (OD) at 620 nm were performed with a Shimadzu spectrophotometer (Kontron Instruments, Italy). The experiments were carried out in the presence of 10 µg of heparan sulphate/fucoidan. OD at 620 increases initially during nucleation phase and decreases during the aggregation phase. Slopes of the nucleation (till the maximum) and aggregation (after the peak) phases were calculated using linear regression analysis and the percentage inhibition of the GAGs were calculated using the formula:

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\text{Percentage inhibition} = (1 - \frac{\text{Sm}}{\text{Sc}}) \times 100, \text{ where}
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Sm is the slope in the presence of the modifier and Sc the slope of the control.
2.2.2. Light microscopic analysis of crystals

Calcium oxalate crystals for light microscopic studies were prepared according to the method of Nakai et al. (1996). Calcium oxalate crystals were formed by mixing 0.2 ml of 20 mM calcium chloride with 0.1 ml of 20 mM potassium oxalate. All the solutions were prepared in 10 mM sodium acetate buffer containing 200 mM sodium chloride and the pH was adjusted to pH 6.5. Crystallization was carried out with and without 20 μg of the respective GAG and incubated for 10 min, after which 50 μl of the formed suspension was spread on a glass slide and a cover slip was placed on it and investigated under light microscope (EC-400 NIKON light microscope) and photographed under 400x magnification.

2.3. RESULTS AND DISCUSSION

Crystallization is a major physico-chemical aspect of calcium renal stone formation that takes place as two discrete steps, a thermodynamic one including urinary supersaturation and nucleation of microcrystals, and a kinetic one comprising of rates of nucleation, growth and aggregation of crystals (Kok and Papapoulos, 1993). Figure 2.1 depicts the crystallization kinetics in the presence of heparin/fucoidan. The time from the addition of calcium upto the first detectable increment of OD at 620 reflects, the time required for calcium oxalate crystal nuclei to form in numbers and grow into size which allows detection. When the slope of increase of OD at 620 with time reaches maximum, the increase in turbidity mainly reflects the increase in particle number as a function of time, and thus crystal nucleation. The slope of decrease of OD at 620 nm after the maximum, is the measure of crystal aggregation. In Figure 2.1, the blue boxes indicates the initial control experiments to assess the reproducibility of the
Figure 2.1. Effect of fucoidan/heparin on crystallization of calcium oxalate

Values are expressed as mean of 6 experiments.
experiments. Pink boxes and the green triangles in Figure 2.1 represent the crystallization kinetic curves in the presence of heparin and fucoidan respectively. Heparin and fucoidan showed 81% and 82% inhibitions on the nucleation phase respectively (Figure 2.2a). Crystal nucleation is an essential prerequisite for further formation of larger particles within the urinary tract, which ultimately may form stones. Fucoidan and heparin were able to profoundly inhibit this nucleation phase by their ability to bind to calcium ions. Fucosylated sulphated polysaccharides from marine algae are known to have calcium binding capacity (Ruggieroa et al., 1994). The anionic groups in the GAGs might be responsible for calcium sequestration as it increases the GAGs affinity towards calcium ions (Cao et al., 1997a). They also postulated that the calcium binding groups in the GAGs might be organized into a macromolecular structure, essential for GAGs to exert its activity. Adding support to this concept, Berteau and Mulloy (2003) also suggested a specific structure for fucoidan which can be linked to its biological effects.

Heparin and fucoidan exhibited 32% and 27% inhibitions on the aggregation phase respectively (Figure 2.2b). Crystal aggregation is probably the major crystallization process that enables stones or kidney calcifications during crystalluria to put up new crystals. It is by means of crystal growth and aggregation that larger particles grow in the urinary tract. The growth of single calcium oxalate crystal is slow to attain a physiologically relevant size, but crystal aggregation allows the formation of large particles at a faster rate. Recurrent stone formers are distinguished from healthy people by the fact that their urines have an abnormal propensity to form large aggregates (Hess and Kok, 1996). In urine, crystals become coated by anionic macromolecules and this electro-negative coat seems to inhibit aggregation by electrostatic repulsion of the individual crystals (Atmani and Khan, 2002; Boeve et al., 1994). The
Figure 2.2a. Effect of fucoidan/heparin on nucleation phase of crystallization

Figure 2.2b. Effect of fucoidan/heparin on aggregation phase of crystallization

Values are expressed as mean ± S.D. of 6 experiments.
inhibitory effect of the GAG, fucoidan on the aggregation phase can be attributed to the potential of the GAG to bind to the growth sites of calcium oxalate crystals (Cao et al., 1997a). Verkoelen et al. (1996) have suggested that marine polysaccharides can act as potential inhibitors of crystallization. Heparan sulphate was also able to protect from calcium oxalate crystallization by inhibiting the degree of aggregation in ultrafiltered human urine (Suzuki and Ryall, 1996). Since, fucoidan a sulphated polysaccharide from marine algae bears similarity with heparin, the inhibitory effect of the fucoidan on nucleation and aggregation phase can be directly correlated with its functional analogue, heparin which is a well known inhibitor of crystallization (Suzuki and Ryall, 1996). Moreover, Senthil et al. (1996) have shown through in vitro studies that SPP, a heparin analogue was an effective inhibitor of crystallization.

Light microscopic analysis of calcium oxalate crystallization under the conditions employed in the present study produced typical COM crystals (Figure 2.3a). On the other hand, in the presence of the fucoidan/heparin, COD crystals were formed predominantly and not the monohydrate crystals (Figure 2.3b and c). Crystalluria is a common event observed even in non-stone forming individuals, and these crystals are predominantly of the COD form (Dyer and Nordin, 1967). In the present study, the major crystalline form found after calcium oxalate precipitation was COM in the absence of the fucoidan/heparin. It has been suggested that COM has a stronger affinity for cell membranes than COD (Lieske et al., 1997) and thus COM crystals may constitute a form of high potential risk for stone formation. Moreover, the most common form of calcium oxalate crystals found in kidney stones is COM (Lieske et al., 1997), although many stones contain both the crystal forms. Thus, fucoidan was able to induce alterations in calcium oxalate crystal morphology by favoring the formation of the COD crystals, which is less likely to bind to renal epithelial cells indicating
Figure 2.3. Light microscopic analysis of crystals (400x)

(a) Aggregated COM crystals formed in the absence of fucoidan/heparin

(b) COD crystals formed in the presence of fucoidan

(c) COD crystal formed in the presence of heparin
its possible preventive role in crystallization. Heparin also formed COD crystals indicating that the fucoidan and heparin have comparable effects.

Data obtained under specific assay conditions in the present study, suggest that fucoidan inhibits the nucleation and aggregation phase of crystallization events. Modulation of crystal kinetics and formation of COD crystals in the presence of fucoidan substantiate the plausible role of fucoidan in lithogenic condition. The logical extension of the present in vitro observation is that this biological polyanionic molecule can play an important role in the prevention of calculi. However, in vivo studies would clearly establish the role of fucoidan for, in complex biological systems, certain potent effectors of lithogenesis in vitro condition might prove neutral. Hence, further studies were oriented to elucidate the role of fucoidan in experimental hyperoxaluria by employing in vivo study models.