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# Urinary Excretion of Glycosaminoglycans in Patients with Urolithiasis

**Seema Jawalekar[1*], Vasant Surve[2] and Anil Bhutey[3]**

*[1]Department of Biochemistry, M.G.M. Medical College, Kamothe, Navi Mumbai, India.*
*[2]Department of Biochemistry, Dr. Ulhas Patil Medical College, Jalgaon, India.*
*[3]Department of Biochemistry, Panjabrao Deshmukh Medical College Amravati, India.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Author SJ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors VS and AB managed the analyses of the study. Authors SJ and VS managed the literature searches. All authors read and approved the final manuscript.*

***Research Article***

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## ABSTRACT

The interaction between tubular epithelial cells and calcium oxalate crystals or oxalate ions is a very precarious event in the lithogenesis. Urine contains ions, glycoproteins and glycosaminoglycans that inhibit the crystallization process and may protect the kidney against lithogenesis.

Hundred patients (24 women and 76 men) with a history of stone disease and hundred controls (25 women and 75 men) were evaluated for urinary GAG concentration. By using a new dye-binding assay, the total GAG concentration in the urine was measured and corrected to urinary creatinine levels (milligram of GAG per gram creatinine).

There was a preponderance of urinary stones in males; the highest incidence being in Group 2. Excretion of GAGs in 24-hour urine sample was significantly lower in patients compared to controls, for males in all age groups and for females, however, there was no statistically significant difference in the urinary GAGs value between males and females in a given age group for either controls or patients. The urinary GAGs excretion idecreased with age in controls, but the levels in patients were lower.

**Conclusion:** This study shows that low urinary GAGs are associated with urinary stones in patients. Our study lead us to propose that urinary GAG may play an important role in

**Corresponding author: Email: seems2april@rediffmail.com;*

the prevention and reduction of calculi in patients with urolithiasis. Lower urinary GAG levels are more common in patients with stone formation. This may play a more determinant role in male patients and those with recurrent stone formation. We conclude that uric acid can constitute an important risk factor for calcium oxalate urolithiasis through heterogeneous nucleation and the GAGs can play an important role as preventive agents.

*Keywords: Calcium; oxalate; glycosaminoglycan; uric acid.*

## 1. INTRODUCTION

The glycosaminoglycans are large unbranched acidic polysaccharides which, with the probable exception of hyaluronic acid, occur attached to a protein core, forming the proteoglycans which form part of the extracellular matrix. Two glycosaminoglycans, chondroitin sulphate and heparan sulphate are excreted free in the urine and can inhibit the nucleation, aggregation and growth of calcium oxalate and uric acid crystals (on which calcium oxalate crystals grow epitaxially).

Urolithiasis is a multifactorial disease. Many aspects of its pathogenesis have been widely investigated. One of them is the interaction between calcium oxalate, the crystal present in most of the human stones, and tubular epithelial cells. The effects of oxalate ion upon tubular cells have also been studied. High oxalate concentrations provide conditions for precipitation of calcium oxalate crystals in the urine, and both calcium oxalate crystal and oxalate ions induce renal injury which generates cellular debris and promotes crystal nucleation [1]. In humans, the main urinary glycosaminoglycans are chondroitin sulfate ( ~ 80% of total) and heparan sulfate ( ~ 20% of total), with small amounts of dermatan sulfate (1% to 2%) and trace amounts of hyaluronic acid and keratan sulfate. There are few studies suggesting that the urinary chondroitin sulfate is of systemic origin and filtered in the glomerulus [2,3] while the urinary heparan sulfate and, possibly, dermatan sulfate could be of systemic origin or come from the kidney and/or urinary tract [2]. There is evidence suggesting that glycosaminoglycans (GAG) are potent inhibitors of growth and aggregation of calcium oxalate crystals in vitro. This finding raises the possibility that the urinary GAG could play an inhibitory role in the urolithiasis. To investigate this hypothesis, a study on the urinary excretion of GAG in normal and stone forming adults were undertaken.

## 2. MATERIALS AND METHODS

Hundred patients (24 women and 76 men) with a history of stone disease and 100 controls (25 women and 75 men). Renal stone patients were selected among those attending the local clinics at A.C.P.M. Medical College, Dhule, Maharashtra (India). One hundred healthy persons of Dhule district (75 males and 25 females), who served as controls, with no recent report of ill health of any kind and had no past history of urolithiasis, including that in the family. The diagnosis of urolithiasis was supported by plain abdominal X–ray, ultrasonography and / or intravenous pyelography. The procedures were approved by the local ethics committee.

The patients had taken no medication for atleast two weeks, and were not suffering from malabsorption, either as a result of tubular acidosis or malformations of the urinary system. Written consent was obtained from patients. 24 hour urine specimens were collected at 4ºC without preservatives. On arrival at the laboratory, the total volume and relative density of

each sample were measured and were evaluated for urinary GAG concentration. By using a new dye-binding assay, the total GAG concentration in the urine was measured and corrected to urinary creatinine levels (milligrams of GAG per gram creatinine).

For GAG measurement, the GAGs were precipitated with cetylpyridinium chloride and then reacted with dimethylmethylene blue to produce a complex with the polyanionic molecule of sulphated GAGs [4]. The GAG results were expressed as a GAG/Creatinine (mg/g, respectively) ratio. Calcium and magnesium, Uric acid, oxalate, and phosphate concentrations were measured by means of conventional enzymatic kits. Statistical analysis was performed using the Student's *t* test for unpaired data.

For purposes of comparison, the Patients were divided into 3 groups: Group 1 (15-30 years), Group 2 (31-45 years) and Group 3 (46-60 years), for either sex. The distribution of the number of subject in each of these groups is shown in Table 1.

## 3. RESULTS

The results are expressed as Mean ± SD and the statistical analysis of the data was performed by students t – test. The analysis was carried out using SPSS 12. P values < 0.05 were regarded as statistically significant.

Urinary excretion of glycosaminoglycans and magnesium measured and the results correlated with sex and age matched control. In normal subjects the daily excretion of magnesium and glycosaminoglycans increased as compared to stone patients. In stone formers there were significant decrease in the inhibitors, like magnesium and glycosaminoglycans and the levels of the stone-forming constituents, such as oxalate, calcium, phosphorus and uric acid are increased significantly.

There was an overall preponderance of urinary stones in males; the highest incidence of urolithiasis was seen between 15 to 45 years of age (group 1,2) (Table 1). We found all study patients to have a relatively higher excretion of calcium, uric acid as compared to controls. The difference was statistically significant. Excretion of GAG in 24-hours urine sample was significantly lower in patients as compared to controls, for males in all Groups. Females in all groups also showed a significantly lower GAG excretion. There was no significant difference in the urinary GAG excretion between males and females in any Group. The urinary GAG excretion versus age showed similar pattern for patients and controls (an increase in GAG excretion with decreasing age), but the levels in stone formers were lower. The mean urinary GAG concentration in those with stones was significantly lower than in the controls ($P = 0.001$).

Table 2 shows Metabolite analysis showed that in patients with urolithiasis 24 hours urine calcium, oxalate, uric acid excretion is higher than normal. Although urine excretions of phosphorus is normal.

**Table 1. Weight distribution and GAG excretion (mean ± SD) among patients and controls**

| | Controls (n = 100) | | Patients (n = 100) | |
|---|---|---|---|---|
| | Males (n = 75) | Females (n = 25) | Males (n = 76) | Females (n = 24) |
| Group 1 (15 - 30 years) | 29 | 05 | 33 | 05 |
| Group 2 (31 - 45 years) | 35 | 12 | 36 | 08 |
| Group 3 (46 - 60 years) | 11 | 08 | 07 | 11 |
| Weight (kg) | | | | |
| Group 1 | 68.2 ± 5.6 | 54.8 ± 6.6 | 71.6 ± 4.6 | 55.6 ± 2.5 |
| Group 2 | 76.3 ± 6.2 | 67.0 ± 5.3 | 79.6 ± 5.5 | 72.0 ± 1.6 |
| Group 3 | 62.6 ± 4.8 | 53.8 ± 4.9 | 61.1 ± 2.9 | 53.7 ± 3.3 |
| 24-hour Glycosaminoglycan excretion mg / gm creatinine | | | | |
| Group 1 | 37.7 ± 9.9 | 35.9 ± 6.9 | 24.6 ± 7.5* | 24.0 ± 4.1* |
| Group 2 | 34.9 ± 7.6 | 33.4 ± 8.7 | 23.5 ± 6.8* | 21.6 ± 8.6* |
| Group 3 | 32.2 ± 9.1 | 31.9 ± 8.2 | 21.8 ± 6.7* | 20.9± 5.6* |

** Statistically significant lower excretion of GAG compared to controls ( P < 0.001).*

**Table 2. Urinary calcium, magnesium, oxalate, phosphate, and uric acid concentrations, and pH values in controls and patients with urolithiasis**

| Parameters | Control ( n=100) | Patients (n=100) |
|---|---|---|
| Calcium | 4.00 ± 0.96 | 11.59 ± 8.00 |
| Phosphorus | 31.80 ± 6.06 | 32.21 ± 8.79 |
| Oxalate | 0.29 ± 0.07 | 0.45 ± 0.19 |
| Magnesium | 3.82 ± 1.72 | 2.38 ± 1.23 |
| Uric acid | 2.58 ± 0.64 | 3.47 ± 1.31 |
| PH | 6.14 ± 0.31 | 6.09 ± 0.64 |

*Concentrations are mean (SD) in mmol/L.*

## 4. DISCUSSION

The interaction between tubular epithelial cells and calcium oxalate crystals or oxalate ions is a very precarious event in the lithogenesis. Urine contains ions, glycoproteins and glycosaminoglycans that inhibit the crystallization process and may protect the kidney against lithogenesis [1].

The mechanisms that are involved in renal stone disease are not entirely clear various concepts proposed by various studies. In this study, attention is dedicated to an glycosaminoglycan that may play a central role in renal stone disease. The precipitation of poorly soluble calcium salts (crystal formation) in the kidney is the inevitable consequence of producing concentrated urine. GAGs are a major constituent of the extracellular matrix in the renal medullary interstitium and the pericellular matrix of mitogen/stress-activated renal tubular cells. GAGs are an excellent crystal-binding molecule because of its size, negative ionic charge, and ability to form hydrated gel-like matrices. Crystal binding to GAGs leads to crystal retention in the renal tubules (nephrocalcinosis) and to the formation of calcified plaques in the renal interstitium (Randall's plaques) [5]. It remains to be determined whether one or both forms of renal crystal retention are involved in the development of kidney stones. Various studies shown that glycosaminoglycans inhibit the growth and aggregation of calcium oxalate crystals in vitro [6,7,8]. The hypothesis of these observations was that the

urinary glycosaminoglycans could function as inhibitors of calcium oxalate urolithiasis. If endogenous glycosaminoglycans are important factors in the inhibitors of urolithiasis, one might expect significant differences in the urinary glycosaminoglycan levels of stone formers in comparison to non stone formers. In fact, our study has shown a significant decrease in the urinary glycosaminoglycan concentration of stone forming subjects.

As GAG excretion was highest in the morning and lowest at night 24 hour urine collection would avoid falsely high results. Therefore, we performed the GAG measurements in 24 hours urine specimens and we also calculated total GAG concentration by using the GAG/Creatinine ratio in 24 hours urine samples, which gives more reliable results [9].

The GAG concentration was increased in the 24-hour urine samples, and in males compared to females though the difference is not statistically significant, the GAG/creatinine ratio was independent of period of urine collection and of sex. So, it was advantageous to express the amounts of urinary GAG as mg/g of creatinine. Group I excreted more GAG than other adults groups. We have shown that the stone forming subjects, all age groups, excreted lower levels of urinary GAG as compared to normal subjects, independently of the metabolic disorder. These results indicate that there is a definite difference in terms of levels of GAG between normal and stone forming urines, and suggest a correlation between the urinary GAG concentration and urolithiasis.

High oxalate concentrations promotes calcium oxalate crystallization and both calcium oxalate crystal and oxalate ions induce tubular injury that could promote crystals nucleation. Tubular cells may protect from the toxic insult by inducing the synthesis of crystallization inhibitors, like glycosaminoglycans [10]. Glycosaminoglycans not only prevent crystallization of calcium oxalate but also prevent uric acid crystallization. Grases et. al in his vitro study found that citric acid and phytic acid caused no effects on uric acid crystallization even at the highest concentrations assayed (1,000 and 5 mg/l, respectively). From the results obtained it can be deduced that mainly glycoproteins, glycosaminoglycans and surfactant substances can exert protective effects against uric acid crystallization [11].

There have been numerous reports showing that urinary GAGs are low in adult patients with nephrolithiasis [12,13,14,15,16]. We also found that urinary GAG concentrations were significantly lower in adults with urolithiasis. However, although Michelacci et al suggested that urinary GAG excretion in children with urolithiasis was significantly lower, [17] these data have not been confirmed by others [18,19].

Our results are supported by Shirane et.al, was found that heparan sulfate and heparin were more effective growth inhibitors than chondroitin sulfate and hyaluronic acid at concentrations within their respective urinary range [20]. With increasing calcium and/or glycosaminoglycans concentration in the solution, the degree of growth inhibition caused by glycosaminoglycans was enhanced. Calcium oxalate crystal shapes generated with various glycosaminoglycans varied with glycosaminoglycan species. One of the causes of those differences in the shape and degree of growth inhibition might be the structural differences between them, that is, the number of sulfate residue and O- or N-form they contain [20].

Urinary GAG concentration is significantly lower in stone-forming patients where as the stone forming minerals like calcium, oxalate, uric acid are significantly increased in stone formers. The present study clearly demonstrates the decreased urinary GAG concentration and excretion in stone-forming patients and suggests an interaction between these inhibitors

and calcium, urate, oxalate that could modify the inhibitory potency of GAGs our findings are concurrent with Nesse et.al [21].

Hyaluronan is not only excellent in retaining water and binding cations [22,23] it also avidly binds crystals [24,25]**.** The synthesis and expression at the cell surface of hyaluronan by developing or regenerating distal renal tubular cells inevitably leads to tubular nephrocalcinosis. Due to its ability to bind calcium ions, hyaluronan is also an excellent inhibitor of crystallization in the renal papillary interstitium. It is tempting to speculate that interstitial nephrocalcinosis can be prevented by increasing the hyaluronan content. Interstitial nephrocalcinosis ultimately leads to Randall's plaques and kidney stones. Increasing hyaluronan in the papillae may therefore also have a beneficial effect on kidney stone prevention [26,27]. Study by Sarica K et.al showed that as the GAG content of the stone matrix decreased, the efficiency of the shock waves on stone disintegration also prominently decreased. The stones which were found to have higher amounts of GAGs in the organic matrix tended to be more fragile and were easily disintegrated with shock waves [28].

## 5. CONCLUSION

This article demonstrates that hyaluronan is likely to be involved in the pathophysiology of all different manifestations of renal stone disease. Better understanding of the factors and mechanisms involved in renal hyaluronan synthesis, deposition and breakdown may lead to more successful treatment strategies.

It is concluded that a higher affinity to the crystals may be the reason why highly charged glycosaminoglycans are more efficient inhibitors of calcium oxalate crystal growth as well as uric acid crystallization.

## CONSENT

All authors declare that ‘written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

## ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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