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# Glycosaminoglycans and other sulphated polysaccharides in calculogenesis of urinary stones

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**Summary.** Naturally occurring glycosaminoglycans (GAGs) and other, semisynthetic, sulphated polysaccharides are thought to play an important role in urolithiasis. Processes involved in urinary stone formation are crystallization and crystal retention. Oxalate transport and renal tubular cell injury are determining factors in these processes. In this article experimental results concerning the possible mechanisms of action of GAGs and other sulphated polysaccharides are reviewed. GAGs are inhibitors of crystal growth and agglomeration and possibly also of nucleation. They can prevent crystal adherence, correct an abnormal oxalate flux and prevent renal tubular cell damage.

Sulphated polysaccharides can be divided in two subgroups, namely, glycosaminoglycans (GAGs) and other semi-synthetic sulphated polysaccharides. GAGs are polyanionic polysaccharide chains composed of repeating disaccharides of identical composition and variable lengths. Their molecular weight varies between $2 \times 10^3$ and $3 \times 10^6$ Da. All have a similar structure with one of the five principal polymers hyaluronate, chondroitin, keratan, dermatan or heparan. All except hyaluronic acid may be covalently attached to protein as proteoglycans [35].

GAGs are widely distributed in the body. Many physiological functions are attributed to these substances, but little is known in detail about their synthesis, distribution and metabolism. In adults, approximately 250 mg GAG is produced each day, only 10% of which is excreted in the urine. Urinary GAGs are enzymatic degradation products of proteoglycans that are excreted by glomerular filtration [50]. Renal tubular secretion or reabsorption has not been demonstrated [37]. Their excretion shows a circadian rhythm. Men excrete significantly more GAGs per 24 h than women, the levels varying between 10 and 30 μmol/day as based upon measurements of the glucuronic acid moiety. Normal urine contains about 2% GAGs. Of the GAG fraction, about 60% is chondroitin sulphate, 18% is keratan sulphate, 15% is heparan sulphate, 4% hyaluronic acid and 2% is dermatan sulphate [29]. Only is heparin does not appear in human urine. Many investigators [5, 14, 17, 25, 33, 61, 64, 65] have studied urinary GAGs, but it remains unclear whether there are qualitative and/or quantitative differences in urinary GAGs between urolithiasis patients and normal individuals. Moreover, we lack detailed knowledge about the role of GAGs in cell membrane function and its influence on cell surface properties.

As early as 50 years ago, Butt [11] used an enzymatic induced hypersecretion of GAGs in the prevention of urolithiasis. This and the introduction of heparin-like drugs and sodium pentosan polysulphate (SPP) induced the interest of investigators to use these substances in stone prevention. Norman et al. [51, 52] first reported on the use of SPP in the treatment of urolithiasis. In humans, approximately 3% of this drug is excreted in the urine after oral administration [58]. Animal studies with tritiated SPP demonstrated a high concentration of the label in rat urine and the urothelium after either i.v. or oral administration [53]. After 30 days of oral administration to rats the total GAG content in kidney tissue had not significantly changed, but a significant increase in the heparin fraction was observed. Changes in other GAG fractions observed during treatment with an induction of a lithogenic diet were prevented with simultaneous SPP administration [71].

In humans after the administration of [$^{125}$I]-SPP, degradation products were found in urine; these occurred in a sulphated and desulphated macromolecular form and in a depolymerized form [45]. However, dermatan sulphate is well absorbed after oral administration but appears unchanged in urine [16]. Other semi-synthetic polysaccharides used in stone research are G871, G872 (both derived from seaweed [7, 13]) and CG-120 [48]. The effect of GAGs on calcium oxalate crystallization in urine has recently been reviewed by Hesse et al. [35] and Cao et al. [12]. In this review we pay extra attention to recently introduced, new possible mechanisms for GAGs in stone

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**Table 1.** The effect of sulphated polysaccharides on crystallization in vitro

| Authors | Reference | Year | Substances | Crystals | | Effect on crystallization | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Nucl. | Growth | Aggr. |
| Robertson et al. | [62] | 1973 | CS-A Heparin | CaOx | SCGM | | Inh. | Inh. |
| Hansen et al. | [32] | 1976 | Heparin HA CS-A, B, C | CaP | | | Inh. | Inh. |
| Bowyer et al. | [9] | 1979 | CS-A CS-C | CaOx | SCGM | | Inh. | Inh. |
| Ryall et al. | [66] | 1981 | CS Heparin | CaOx | SCGM | | Inh. | Inh. |
| Drach et al. | [18] | 1982 | Heparin | $CaOx2H_2O$ | MSMPR | Prom. | Inh. | |
| Martin et al. | [44] | 1984 | SPP | CaOx | SCGM | | Inh. | |
| Norman et al. | [52] | 1985 | SPP | CaOx | MSMPR | | Inh. | Inh. |
| Fellström et al. | [19] | 1985 | Heparin SPP | CaOx | SCGM | | Inh. | |
| Kok et al. | [39] | 1988 | CS Heparin SPP | $CaOxH_2O$ | SCGM | | Inh. | No effect |
| Danielson et al. | [15] | 1989 | SPP | CaOx | SCGM | | Inh. | |
| Kohri et al. | [38] | 1989 | CS | CaOx | MSMPR | Inh. | Prom. | |
| | | | HA Heparin | CaOx | MSMPR | Prom. | Prom. | |
| Osswald et al. | [54] | 1989 | CS-A, B, C SPP | CaOx | MSMPR | Inh. | Inh. | |
| McLean et al. | [46] | 1990 | CS-A, C Heparin | Struvite | ISM | No effect | No effect | |
| Grases et al. | [30] | 1991 | CS SPP | UA | Turb. | Inh. | Inh. | Inh. |
| Miyazawa et al. | [48] | 1991 | CG-120 SPP | CaOx | MSMPR | Inh. | | |
| | | | | | SCGM | Inh. | Inh. | Inh. |
| Cao et al. | [13] | 1992 | SPP G871 G872 | $CaOxH_2O$ | CCM | | Inh. | Inh. |
| Boevé et al. | [7] | 1993 | SPP G871 G872 | CaP | CCM | | Inh. | Inh. |

*Nucl.,* Nucleation; *Aggr.,* aggregation; *CS,* chondroitin sulphate; *HA,* hyaluronic acid; *CaP,* calcium phosphate; *UA,* uric acid; *SCGM,* seeded crystal growth model; *MSMPR,* mixed suspension, mixed product removal system; *ISM,* infection stone model; *Turb.,* turbidity measurement; *Inh.,* inhibition; *Prom.,* promotion

formation/prevention. The mechanisms by which GAGs can influence crystallization and stone formation in urine are:

1. Inhibition of crystal nucleation, growth and aggregation
2. Prevention of crystal adherence
3. Correction of abnormal oxalate transport
4. Protective effect on renal tissue

## Inhibition of crystal nucleation, growth and aggregation

Crystal nucleation, growth and aggregation depend on supersaturation and on crystallization inhibitors and promoters. Crystallization inhibitors can be divided into low- and high-molecular-weight substances. GAGs and the semisynthetic sulphated polysaccharides belong to the high-molecular-weight inhibitors. In contrast to low-molecular-weight inhibitors, they can inhibit crystallization in very low concentrations. This effect can be explained only by a direct interaction of the polysaccharides with the crystals, blocking their growth sites and/or changing the crystal surface properties.

Absorption on calcium oxalate crystals was demonstrated by Leal and Finlayson [41] with the solution depletion method. They observed a binding of heparin (63%) and chondroitin sulphate (70%). Later they found with the same method a $Ca^{2+}$- and $Mg^{2+}$-dependent adsorption of heparin on sodium acid urate crystals [22]. Fellström et al. [20] confirmed these data with binding experiments with radiolabeled heparin, chondroitin sulphate and SPP. Under a condition of an excess of sodium urate crystals, up to 90% of the offered chondroitin sulphate was bound, whereas only 40% of the SPP was bound. None of the polysaccharides used showed binding to uric acid crystals under similar conditions. In 1989, Fellström et al. [21] showed a very high affinity for the same polysaccharides to calcium oxalate crystals. Binding was not influenced by the pH of the solution, but a lower binding affinity at a higher ionic strength was observed. Angell and Resnick [1] applied the Langmuir isotherm absorption method to study the surface interaction between calcium oxalate and GAGs. Components known to be weak inhibitors bind with less affinity than strong inhibitors. Zeta-potential measurements provided evidence for the binding of SPP and two newly developed sulphated polysaccharides, G871 and G872, to calcium oxalate monohydrate [13] and calcium phosphate crystals [7]. The highly negatively charged polysaccharides reduce the zeta potential of the crystals, the effect being more pronounced with G872 than with G871 or SPP.

The effect of sulphated polysaccharides on crystallization has been studied in vitro by many investigators using different crystallization models with different crystals, different media and different inhibiting substances (Table 1). The majority of the investigators have found that crystal growth and aggregation is inhibited to various degrees. The results obtained in different model systems are not

comparable and may or may not be considered representative for the processes occurring in the human urinary system. We tested all available GAGs and semi-synthetic polysaccharides in a constant composition system and a seeded crystal system (unpublished data). According to our results, the relative inhibitory activity on calcium oxalate crystal growth is: semi-synthetic sulphated polysaccharides > heparin > chondroitin sulphate. Several factors must be considered that can influence the results obtained, including (a) the total polysaccharide mass bound to the crystal surface, (b) the molecular weight, (c) the site and degree of sulphation of the polysaccharides and (d) the existing state of the polymers in solution.

The effect of the different polysaccharides on crystals other than CaOx is less well established. Several investigators have shown that only low-molecular-weight substances such as citrate, magnesium, phosphocitrate, pyrophosphate and diphosphonates can inhibit calcium phosphate crystallization. Hansen et al. [32] and Boevé et al. [7] showed inhibition of calcium phosphate crystallization by heparin, hyaluronic acid, SPP and G872. We did not find a report on the inhibition of uric acid growth or aggregation. Recently McLean et al. [46] showed no effect of sulphated polysaccharides on struvite formation.

Colloidal monosodium urate was believed to reduce the inhibitory activity of urinary GAGs in calcium oxalate urolithiasis with hyperuricosuria [63, 67]. Recently, Grover et al. [31] concluded that "urate does not exist in urine in a colloidal or crystalline form, and that the promotion of calcium oxalate crystallization by urate is not a consequence of its reducing the inhibitory activity of GAGs".

Butt [11] introduced the idea that urinary colloids might be active in the prevention of urolithiasis. The highly sulphated and therefore highly negatively charged polysaccharides are supposed to bind to the urinary crystals and in that way stabilize the urinary colloidal system. According to the protective colloidal theory, crystal aggregation will be inhibited with an increase of the crystal surface charge. The inhibitory activity of different sulphated polysaccharides is related to their surface charge, as has been elegantly demonstrated with the zeta-potential measurements of Robertson et al. [62] and Cao et al. [13].

Apart from the GAG-crystal interaction, there is evidence that urinary polysaccharides can inhibit calcium oxalate crystallization by lowering the urinary supersaturation. Hesse et al. [34] found with an equilibrium analysis that 1 μmol chondroitin sulphate can bind 0.76 μmol calcium. This binding can, dependent on the pH value, be inhibited by urate ions to a maximum of 31%. We do not think that this effect will have a significant impact on the urinary supersaturation, since the concentration of urinary GAGs can exert only little effect, if any, on the urinary calcium concentration.

In an animal experiment using a lithogenic diet with sodium glycolate in the rat, Subha and Varalakshmi [70] have found that after oral administration of SPP, the urinary oxalate and calcium excretion decreases by 25% and 20%, respectively. The glycolate-induced hyperoxaluria is reduced by 20% after oral administration of SPP. They also found a moderate increase in urinary magnesium levels after 30 days of SPP administration. However, Michelacci et al. [47] did not find a protective effect on experimentally induced calcium oxalate crystallization in the rat bladder following intraperitoneal administration of chondroitin sulphate to rats.

GAGs can be found in matrix from stone. Heparan sulphate is the main GAG occurring in soluble stone matrix [73]. It can be speculated as to whether GAGs in the stone matrix are promoters of crystallization of whether they are inhibitors adsorbed to the crystal surface.

## Prevention of crystal adherence

Crystallization alone is insufficient to explain the occurrence of urinary stones. Adherence of crystals to the urothelium is an additional prerequisite for stone formation, as Finlayson et al. [23] have suggested. These authors calculated that the transition time of urine is too short for free crystals to grow into urinary stones. Therefore, it is important to study the interaction of crystals with mucosal surfaces. In fact, not only urolithiasis but also urinary tract infection and bladder cancer may be mediated by the adherence to the urothelium of, respectively, crystals, bacteria or carcinogens.

The normal urothelium is lined with a GAG layer [49]. Parsons and co-workers [56, 57] showed in a good experimental set-up that the GAG layer, produced by transitional cells lining the rabbit bladder, prevented bacterial adherence. When this GAG layer was removed by protamine sulphate treatment, bacterial adherence increased. Exogenous sulphated polysaccharides can reverse the protamine-induced changes. Gill et al. [27] demonstrated the same effect on calcium oxalate crystal adherence. They showed an elevation of the metastable limit for nucleation and an absence of nucleation on the reaction container surface in a urothelium-lined system. When the urothelium was injured with a detergent there was a marked increase in crystal adhesion [26]. Heparin had a pronounced effect in restoring the normal crystal adhesion properties of the injured urothelium. Chondroitin sulphate-C and hyaluronic acid had no protective effect [28]. Smith [69] demonstrated that chondroitin sulphate and heparin fully restored the anti-adherence properties of the urothelium after acid treatment, whereas SPP caused only a partial restoration.

The above-mentioned experimental models in vivo show the importance of an intact epithelial membrane. Sulphated polysaccharides are a very important constituent of this membrane. They create a highly organized, impermeable water layer that might be responsible for the prevention of crystal adherence.

Recently, more investigators have realized the importance of the role that renal tubular cells play in initial stone formation. The concept that cellular injury and dysfunction are underlying causes of urolithiasis has been accepted. Mandell et al. [43] studied the crystal-membrane interactions with red blood cells in vitro. The membranolysis induced by weddellite and sodium urate was inhibited after the addition of chondroitin sulphate-A, chondroitin

sulphate-C and heparin. Later, Riese et al. [60] developed a cell culture model of renal papillary collecting tubule cells in which they demonstrated cell injury (loss of cell membrane polarity induced by ethylene glycol tetraacetic acid, EGTA) enhanced calcium oxalate crystal adherence. GAGs have not yet been tested in this model.

More recently, crystal adherence studies have been carried out in our laboratory using renal tubular cell lines. Verkoelen et al. [72] demonstrated a time- and concentration-dependent adhesion and/or endocytosis of $^{14}C$-baleled calcium oxalate monohydrate crystals to monolayers of MDCK and LLC-PK1 cells. It was demonstrated that monolayers with a higher degree of differentiation were better protected against crystal adherence. Pretreatment of the crystals with sulphated polysaccharides prevented crystal adherence, in contrast to pretreatment of the monolayer with these compounds. In general, semisynthetic polysaccharides are more effective in preventing crystal-cell interactions than are GAGs [72]. These cell models will be used to study the interaction between crystals and renal tubular cells in more detail. Primary cultures of proximal and distal renal tubular cells are needed for more conclusive results.

Parsons et al. [59] have found an inhibition of sodium urate crystal adherence to the bladder surface by SPP. They have also demonstrated that in the experimental setting, pretreatment of the crystals is more effective than pretreatment of the mucus-deficient bladders. Their results have been confirmed by Pantazopoulos et al. [55]. One can speculate that the previously well-documented interaction between polysaccharides and urinary crystals is of more importance in stone prevention than an intact GAG coating of the urothelium. The GAG coating produced by the cells is a very highly organized layer. The GAGs added to the culture medium or the bladder cannot easily be incorporated in this layer.

Recently, Lieske and Toback [42] found evidence that calcium oxalate monohydrate, brushite and hydroxyapatite crystals are endocytosed by MDCK and BSC-1 cell lines. Heparin is one of the substances inhibiting this process. These investigators think that the surface of the cell, rather than the crystal, appears to be the locus at which heparin and other substances act to inhibit endocytosis. Crystal endocytosis could be an important pathogenic step in urolithiasis.

Future studies will have to reveal whether increased crystal adherence to cells is caused by a defective GAG layer on the cell surface or whether the crystals themselves induce cell dysfunction (by endocytosis?), resulting in an abnormal surface coating. Is there secretion of GAGs by renal tubular cells and, if so, is this of importance in stone prevention?

## Correction of abnormal oxalate transport

Hyperoxaluria is now considered to be the most important risk factor in calcium oxalate urolithiasis. Renal leak, intestinal hyperabsorption or high degrees of endogenous oxalate production can cause high levels of oxalate excretion. It is unknown as to whether or not there is a common (inherited) factor involved in idiopathic calcium oxalate urolithiasis.

Baggio et al. [3] reported an increased oxalate flux across red-cell (RBC) membranes in calcium nephrolithiasis patients. They speculated that in idiopathic urolithiasis patients there may be a genetic cellular defect or membrane disorder causing the increase in oxalate transport. This could also involve the renal oxalate handling. The defect can be corrected by administration of a mixture of heparan and dermatan sulphate [4]. These investigators demonstrated an increased phosphorylation of anion transporters (band 3 protein) in RBC ghosts derived from urolithiasis patients that may result in a high level of oxalate transport. This phosphorylation can be reduced by treatment with GAGs.

## Protective effect of GAGs on renal tissue

There is increasing evidence that some form of renal tubular disfunction such as abnormal renal handling of various ions [68] might be related to tubular damage (as evidenced by enzymuria) [2, 36] and be involved in urolithiasis. Recently, Subha and Varalakshmi [70] found a significantly increased urinary excretion of enzymes (lactate dehydrogenase, LDH; alkaline phosphatase; γ-glutamyl transpeptidase, γ-GT, and β-glucuronidase) in rats treated with a stone-inducing diet containing sodium glycolate. This finding indicates proximal tubular damage and membranuria. SPP treatment normalized the excretion of LDH and moderately decreased that of alkaline phosphatase, γ-GT and β-glucuronidase.

In animal models, crystal formation can be induced by renal tubular injury with gentamycin sulphate or ammonium chloride with ethylene glycol [8, 40]. A variety of ultrastructural changes appear in the proximal tubular cells. Also, a dilatation of the proximal renal tubules occurs and, later, intracellular crystals appear; these have been proven to be calcium oxalate monohydrate crystals [10]. In this animal model, orally applied exogenous sulphated polysaccharides G872 and SPP can prevent the described tubular cell injury [6].

The long-term administration of heparin and dermatan sulphate can also prevent morphological renal alteration and albuminuria in diabetic rats [24]. Abnormal GAG metabolism could determine the loss of glomerular basement membrane anionic charges. Gambaro et al. [24] demonstrated in rats with streptozotocin-induced diabetes that daily s. c. injections of heparin and dermatan sulphate increased the glomerular anionic charge and prevented glomerular basement membrane thickening. The glomerular filtration rate did not decrease and albuminuria did not occur.

## Open questions

In the past 20 years, much effort has been invested in discovering the cause of urolithiasis. Several test systems in vitro and in vivo have been developed. It is now clear that supersaturation is not the only factor that should be taken into account. The lack of inhibitory activity might be the

most important risk factor in urolithiasis. GAGs are probably among the strongest inhibitors of crystallization. We think that it is of great importance that the factors influencing the role of GAGs in urolithiasis be clarified in detail:

1. Is there a difference in urinary GAGs between stone-formers and healthy persons in synthesis, metabolism, molecular structure or conformation in urine?
2. Which individual GAG is an inhibitor and which is a promoter of crystallization?
3. What is the mechanism of crystal adherence and crystal endocytosis? How are these processes influenced by sulphated polysaccharides?
4. Can semi-synthetic polysaccharides prevent renal tubular cell damage in stone patients?
5. Is it possible to prevent stone recurrence by treatment with semi-synthetic sulphated polysaccharides?
6. Can the function of semi-synthetic polysaccharides be improved by increasing the degree of sulphation or changing the molecular structure?

To address these and numerous other questions remaining open, ongoing research will have to focus on both the urinary and the cellular processes involved in stone formation. Such research will answer some questions but will probably put forward new ones as well.

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