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The Effects of Glycosaminoglycans, Pyrophosphate and Allopurinol Treatment on Urease-Induced Crystallization in vitro

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Summary. Previous studies have found that human urine inhibits urease-induced crystallization in synthetic urine. To identify the inhibitory components the effects of chondroitin sulphate, heparin and pyrophosphate on urease-induced crystallization were studied and the inhibitory capacity of human urine collected during and before/after allopurinol treatment compared. None of the substances studied nor allopurinol treatment was found to influence the urease-induced crystallization. These findings do not support the idea that glycosaminoglycans or pyrophosphate are responsible for the inhibitory effect detected in human urine.

Key words: Urease-induced crystallization, Urinary tract concrements, Glycosaminoglycans, Pyrophosphate, Allopurinol.

Introduction

Urine contains several macromolecules: uromucoid, stone-matrix, glycosaminoglycans (GAGS), polyribonucleotides and peptides. Their role in the pathogenesis of calcium-stone formation has received much attention and there is strong laboratory evidence which suggests that GAGS are potent inhibitors of calcium oxalate crystal growth and aggregation [18]. The significance of GAGS in calcium phosphate crystallization is less well established and their role in urease-induced crystallization formation is unknown. The urinary GAGS concentration is markedly diminished in patients with calcium phosphate – magnesium ammonium phosphate calculi, which suggests that GAGS may be involved primarily or secondarily in the process of urease-induced concrement formation [1]. Pyrophosphate has also been considered to play a role in urinary tract concrement formation as an inhibitor of calcium phosphate transformation [4] and crystal growth [3]. More recent studies, however, have failed to demonstrate any effect of pyrophosphate on calcium phosphate crystal formation [8].

In a recent experimental study [6] human urine was shown to inhibit urease-induced crystallization of calcium phosphate and magnesium ammonium phosphate (struvite) when added to synthetic urine in vitro. In a effort to identify the inhibitory component(s) of human urine, the influence of GAGS and pyrophosphate on urease-induced crystal formation in synthetic urine was investigated. The inhibitory effect of urine collected before or during allopurinol treatment was also compared. GAGS' inhibitory effect on urine crystallization is reduced by the presence of urate [14, 15]. As allopurinol decreases the urinary excretion of uric acid, allopurinol treatment could be expected to reduce the inhibitory effect of human urine on in vitro crystallization if the GAGS were responsible for this effect.

Material and Methods

Synthetic urine, 250 ml, prepared according to Griffith et al. [7] was inoculated with urease (Jackbean urease, E.C. 3.5.1.5, 7 units · mg^{-1}, 1 unit = 1 mg NH_3 · 5 min^{-1} at pH 7 and +30 °C, Sigma Chemical Co., USA) dissolved in 0.25–0.5 ml 0.1 M tris buffer, pH 7.2. The incubation was performed under sterile conditions in eight sealed glass vessels placed together in a water bath maintained at +37 ° for 4 h during continuous stirring. Each reactor harboured four glass rods immersed in the synthetic urine. The ammonium ion concentration and pH of the solution were measured at the start and at the end of the incubation. The urease activity was reflected by the ammonium ion increase. Crystallization was studied as the amount of encrustation on the glass rods after the incubation.

In the first set of experiments four of the eight vessels were inoculated with only synthetic urine and urease and served as controls. To the other four reactors was added either chondroitin sulphate (as sodium salt preapred from whale cartilage, Sigma Chemical Co., USA, final concentration 0.4 mg · l^{-1}), heparin (as sodium salt prepared from porcine intestinal mucosa, final concentration 0.4 mg · l^{-1}) or pyrophosphate (final concentration 0.5 mg · l^{-1}). Urease was added in two concentrations; 0.01 and 0.02 units urease · ml^{-1}. Three experiments were performed for each urease concentration and each substance tested.

Table 1. The effects of heparin, chondroitin sulphate and pyrophosphate on urease-induced crystallization in synthetic urine (mean ± S.D. of three sets of experiments)

	0.01 units urease · ml^{-1}			0.02 units urease · ml^{-1}		
	Final pH	Amount precipitated (μg ± S.D.)		Final pH	Amount precipitated (μg ± S.D.)	
		Mg^{2+}	PO_4^{3-}		Mg^{2+}	PO_4^{3-}
0.04 mg · l^{-1} heparin	7.95 ± 0.04	80 ± 15	1,100 ± 180	8.75 ± 0.01	29 ± 6	420 ± 29
controls	7.92 ± 0.03	80 ± 15	1,130 ± 110	8.75 ± 0.01	27 ± 2	410 ± 22
0.4 mg · l^{-1} chondroitin-sulphate	7.80 ± 0.08	100 ± 10	1,330 ± 170	8.67 ± 0.03	29 ± 2	440 ± 27
controls	7.80 ± 0.09	120 ± 10	1,350 ± 80	8.68 ± 0.01	31 ± 3	460 ± 21
0.5 mg · l^{-1} pyrophosphate	7.83 ± 0.06	90 ± 10	910 ± 190	–	–	–
controls	7.78 ± 0.02	90 ± 10	838 ± 100			

A daily oral dose of 300 mg Zyloric^R (allopurinol) was given for 18 days to a 38-year-old male without a history of renal disease. Morning urine was collected on each of the last eight days of the treatment. Morning urine samples were also obtained on each of the four days before the allopurinol administration and another four morning samples were obtained ten days after the allopurinol treatment. The uric acid concentration was measured in all samples. The urine was prepared as described by Lanzalaco et al. [12] and stored at +4 °C before use. Two experiments were performed. In each experiment four reactors were primed with human urine collected during allopurinol treatment and the other four reactors with human urine collected before/after allopurinol treatment. In one experiment 12.5 (5%) ml of human urine and 0.02 · ml^{-1} units of urease was added to the synthetic urine. In the other experiment 5 (2%) ml of urine and 0.01 · ml^{-1} units of urease were added to the synthetic urine.

After each experiment the rods were extracted and dried at +40 ° for 2 h and the encrusted material was dissolved in 0.2 ml 15 HNO_3. From this solution, magnesium was analyzed by atomic absorption spectrometry [16] and phosphate was analyzed using a colorimetric method [19]. The results were calculated as the mean ± S.D. of the 4 rods in each vessel. The amount of magnesium precipitated was assumed to be present as struvite, the non-struvite bound phosphate being assessed as other phosphates (calcium phosphates) as has been shown previously [10].

The pH determinations were performed using a precision pH-meter and a surface pH electrode (model 701A and electrode 9–35, Orion Research Inc., USA). The ammonia and ammonium ion and the uric acid measurements were performed using commercial kits (Sigma kit No 640-A and 292-UV, Sigma Chemical Co., USA). The ammonia and ammonium ion concentrations were measured as a sum. Results are given as mean ± S.D.

Results

Inoculation of pure synthetic urine with 0.01 units urease · ml^{-1} caused a pH increase to 7.78–7.95 in the different experiments (Table 1). The inoculation with 0.02 units of urease · ml^{-1} gave a greater pH increase, to 8.68–8.75. The variation in the pH obtained between vessels incubated simultaneously in the same experiments was very small (coefficient of variation of 0.9%). The addition of heparin, chondroitin sulphate or pyrophosphate did not influence the urease activity measured as pH-increase (Table 1) or ammonium ion concentration increase.

Inoculation with 0.01 units urease · ml^{-1} caused a more pronounced encrustation compared to the inoculation with 0.02 units of urease · ml^{-1}. Neither the addition of heparin, chondroitin sulphate nor pyrophosphate altered the precipitation of magnesium or phosphate on the rods (Table 1).

Allopurinol treatment reduced the mean urinary uric acid concentration from 0.38 ± 0.12 mg · ml^{-1} to 0.26 ± 0.11 mg · ml^{-1} in the 8 urine samples collected during allopurinol treatment. The phosphate concentration in all 16 samples was 2.3 ± 0.6 mg · ml^{-1} with a range of 1.4–4.3 mg · ml^{-1}.

When 12.5 ml (5%) human urine and 0.01 units of urease was added the pH increased to 7.93 ± 0.04. When human urine obtained during or before/after allopurinol treatment was added there were no differences in urease activity, pH increase, the encrustation of phosphate (1,040 ± 130 μg and 1,010 ± 130 μg respectively) or magnesium (46 ± 5 and 46 ± 5 μg respectively). In the second experiment when 5 ml (2%) human urine and 0.02 units of urease were added the pH increased to 8.64 ± 0.04. Also under these conditions there were no differences in urease activity, pH increase, precipitation of phosphate (620 ± 120 μg and 600 ± 110 μg respectively) or magnesium (40 ± 14 and 41 ± 12 respectively) when human urine obtained during or before/after allopurinol treatment was added.

Discussion

The results obtained do not support the hypotheses that heparin, chondroitin sulphate or pyrophosphate influence urease-induced crystallization, or that a reduction in the uric acid excretion influences the inhibitory effect of human urine on urease-induced crystallization.

Crystallization of calcium phosphates from supersaturated solutions in vitro starts with the formation of an amorphous precipitate. This material undergoes transformation to crystal calcium phosphates, hydroxyapatites, which by a process of crystal growth and aggregation lead to the formation of visible concrements. GAGS (heparin and hyaluronic acid) are known to dealy the transformation [13] as well as the crystal aggregation [9]. Sutor et al. [17], however, did not find that urine contained macromolecules which could influence the formation of calcium phosphate concrements.

A large number of experimental models have been used to study crystallization in urine, and they have sometimes given rather contradictory results. One problem with in vitro experiments is whether their results are relevant for the in vivo situation. We have used the experimental model of the present study and found human urine to inhibit urease-induced crystallization in synthetic urine [6]. In the present study pyrophosphate and GAGS were added in the same amounts that are present in the volumes of human urine (10%) which we have found to give a marked inhibition of the urease-induced crystallization. The normal concentration of the urease-induced crystallization. The normal concentration of both GAGS and pyrophosphate in human urine is approximately 5 $mg \cdot l^{-1}$ [1, 2]. As we failed to obtain any reduction in crystallization with these amounts of GAGS and pyrophosphate using the same experimental model it appears unlikely that the reduction obtained with human urine is due to GAGS or pyrophosphate.

Although heparin is not present in urine it was studied because an inhibitory effect has been attributed to it in other studies [18]. No effect of heparin, however, was obtained in this study.

Urate has been claimed to attenuate the inhibitory effects of GAGS [14, 15]. Other recent studies, however, have questioned the role of uric acid in calcium stone formation [5]. The present study did not reveal any difference between the effects of human urine obtained during allopurinol treatment and human urine with a normal uric acid concentration, which indirectly confirms the fact that GAGS do not influence urease-induced crystallization. The results obtained in the present study, however, do not rule out the possibility that GAGS other than those studied may influence this form of crystallization, a possibility supported by the fact that allopurinol treatment reduces the precipitation of calcium phosphates and struvite in patients with indwelling urethral catheters [11].

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