Urol Res (1993) 21:363–366

Urological Research

The effects of citrate and urine on calcium oxalate crystal aggregation

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Received: 6 March 1993 / Accepted: 28 May 1993

Summary. The rate of crystal sedimentation in a suspension of calcium oxalate monohydrate (COM) crystals was determined spectrophotometrically in the presence and absence of dialysed urine and citrate. A reduced rate of crystal sedimentation after stirring was recorded in suspensions containing citrate in concentrations between 0.33 and 1.67 mmol/l. The sedimentation rate was reduced in the presence of a 0.3–3.3% concentration of dialysed urine, with increased inhibition of crystal sedimentation when the concentration of urine was increased. A comparison of the inhibition of COM crystal sedimentation in whole urine and in dialysed urine from normal subjects and stone-formers disclosed significantly higher values ($P < 0.05$) in the dialysed urine. The results support previous observations that physiological concentrations of citrate might efficiently inhibit the aggregation of COM crystals. Furthermore even low concentrations of both whole urine and dialysed urine are apparently very efficient inhibitors of COM crystal aggregation.

Key words: Calcium oxalate – Citrate – Crystal aggregation – Dialysed urine – Inhibition – Sedimentation rate

A low urinary excretion of citrate is a common finding in patients with recurrent calcium stone formation [1–5]. Administration of alkali corrects this abnormality and treatment with alkaline citrate has in several studies proved to be efficient in reducing the risk of stone formation [6–8].

Citrate might influence the crystallization properties in urine in several ways. Complex formation between citrate and calcium ions decrease the ion-activity products of calcium oxalate as well as of calcium phosphate. The resulting change in supersaturation brings about a reduced potential of crystal formation and crystal growth. It has also been demonstrated that citrate is capable of directly inhibiting the growth rate of both calcium oxalate crystals [9] and calcium phosphate [10]. This effect has usually been demonstrated in crystallization systems containing low concentrations of citrate either in the absence of urine or in very diluted urine samples, but the importance of this effect in undiluted urine is less obvious [11].

Aggregation of crystals appears to be of crucial importance in the formation of a renal stone and it has been suggested that the most pronounced discriminating factor between stone-forming and non-stone-forming urine might be the capacity of urine to inhibit crystal aggregation [12, 13]. It is, however, difficult to assess crystal aggregation directly because of the simultaneous growth and aggregation that occur in supersaturated solutions, and the information in this respect is therefore limited.

Kok and coworkers [12] presented strong evidence for a direct effect of citrate on the aggregation of calcium oxalate crystals. They based their conclusion on observation of crystallization kinetics in a metastably supersaturated system seeded with COM crystals and supported these results with laser flow cytometric analysis of the crystal size distribution. In contrast Hess and coworkers [14], who studied the sedimentation rate of COM crystals in the presence of different urine constituents, were unable to demonstrate a direct effect of citrate on crystal aggregation. On the other hand urinary macromolecules proved to be efficient inhibitors of aggregation.

The experiments presented in this paper were undertaken in an attempt to study further the role of citrate and a fraction of urinary macromolecules on COM crystal aggregation.

Materials and methods

The effect of different urine constituents on the rate of crystal sedimentation was measured according to the principles described by Hess and coworkers [14]. A crystal suspension was prepared by adding 300 mg of calcium oxalate monohydrate (COM) crystals (BDH, Kebo AB, Stockholm) to 100 ml of a solution containing 10 mmol/l TRIS buffer and 90 mmol/l sodium chloride. The suspension was carefully mixed by magnetic stirring during 1 h, after which the pH was adjusted to 7.2. The suspension was subsequently placed in an ultrasound bath for 60 min and stored at least overnight at a temperature of 37 °C. Before its experimental use the pH was again checked and adjusted when necessary. Finally the suspension was subjected to ultrasonication for 30 min.

For studies on crystal sedimentation 0.5 ml of the test solution (citrate, dialysed urine or whole urine) was added to 14.5 ml of the crystal suspension. Preincubation was carried out in a shaking apparatus (LabLine, Ninolab, USA) at 150 rpm and 37 °C for 2 h. The suspension was then immediately transferred to a cuvette in a spectrophotometer (Lambda 2, Perkin-Elmer, Überlingen, Germany). Magnetic stirring

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was continued at room temperature in the cuvette for 3 min, during which period a stable absorbance at 690 nm was recorded. The sedimentation of crystals started as soon as the magnetic stirring was stopped. The rate of sedimentation was followed by continuous recording of the absorbance during the following 350 s. A reduction in the sedimentation rate compared with that in the pure TRIS buffer saline suspension was assumed to reflect an inhibition of crystal aggregation [4, 5]. The inhibition in suspensions containing different samples was expressed as the percentage reduction of the absorbance recorded after 300 s in comparison with the absorbance in a reference suspension containing 0.5 ml of 0.15 mol/l sodium chloride. The reproducibility of repeated measurements was very good.

Dialysis of urine was carried out as follows: Spectrapore no. 3 dialysis tubings, with a molecular cut-off at 3500 Da, were filled with 100 ml of urine and stored cold overnight in glass beakers containing deionized water with a volume 10 times that in the dialysis tubing. During the following day (at room temperature), the water was exchanged eight times and during day two, three times with water prepared in a Milli-Q water system (Millipore, USA) and three times with 0.15 mol/l sodium chloride. Continuous magnetic stirring was used during the preparation period. The original volume was re-established by addition of saline up to 100 ml.

Solutions of sodium citrate were prepared to give citrate concentrations in the final suspension corresponding to those in urine. All chemicals used were of analytical grade.

Particle size distribution was determined, after necessary dilution with saline, in a Coulter counter in the following size intervals: 2.8–4.5, 4.6–5.4 and 5.5–14 μm.

We also measured the rate of crystal sedimentation in samples of whole urine and dialysed urine from 13 normal men (NM), 14 normal women (NW), and 35 men (SFM) and 11 women (SFW) with calcium stone disease. The different groups, who had similar age distributions, collected the urine between 2200 and 0600 hours in bottles containing 10 ml of 3 mmol/l sodium azide.

Group comparison was carried out with Student's *t*-test on paired samples.

Results

The sedimentation rate expressed as the percentage optical density relative to that at the starting point, when magnetic stirring of the suspension was stopped, is shown for saline and four different samples of dialysed urine in Fig. 1. The diagram demonstrates a much steeper slope for the suspension without urine. There was a pronounced effect despite a concentration of urine of only 3.3%. Sedimentation of crystals in the system was detected as early as 50 s after turning off the magnetic stirring. An expression of the relative sedimentation rate was obtained by comparing the absorbance in the suspension containing the sample with the absorbance in the reference suspension, which contained saline instead of urine.

The effect of 0.3–3.3% of dialysed urine on the sedimentation of crystals is shown in Fig. 2, where it is evident that increased concentrations of dialysed urine were associated with an increased retardation of the rate of crystal sedimentation. A sharp increase for this particular urine was recorded when the concentration of dialysed urine added to the system was increased from 1.2 to 1.5%.

Addition of citrate in different concentrations also affected the crystal sedimentation. The absorbance at different times after stopping the magnetic stirring is shown in Fig. 3. Although a low citrate concentration of 0.83 mmol/l resulted in a sedimentation rate below that recorded for the reference suspension with saline, citrate in concentrations of 1.7, 2.8 and 3.3 mmol/l all reduced

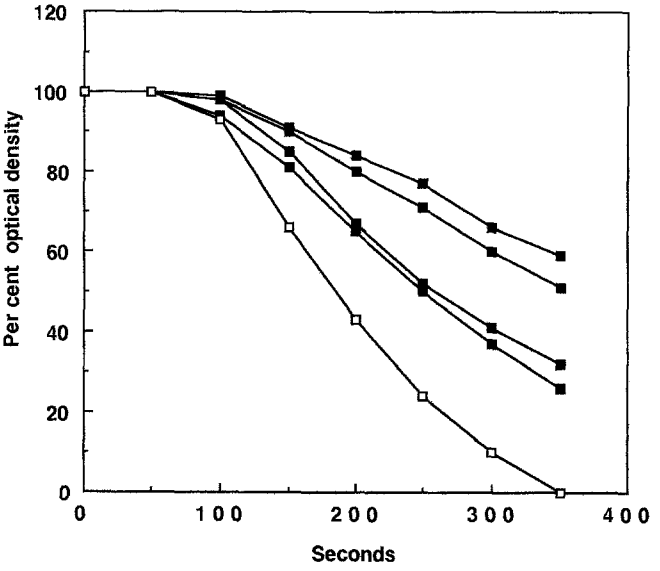

Fig. 1. The reduction in optical density in a suspension of 3 mg/ml calcium oxalate monohydrate (COM) crystals without (□) and with (■) different samples of dialysed urine during the first 350 s after interruption of stirring. The measurements were carried out at a wavelength of 690 nm and with a sample concentration of 3.3% (v/v)

the rate of crystal sedimentation. There was also a positive relationship between the concentration of citrate and the inhibiting activity, with values of −7, 11, 27 and 48% for citrate concentrations of 0.8, 1.7, 2.8 and 3.3 mmol/l, respectively.

Analysis of the crystal size distribution in the suspension before use in the sedimentation experiment showed in four different measurements on four consecutive days

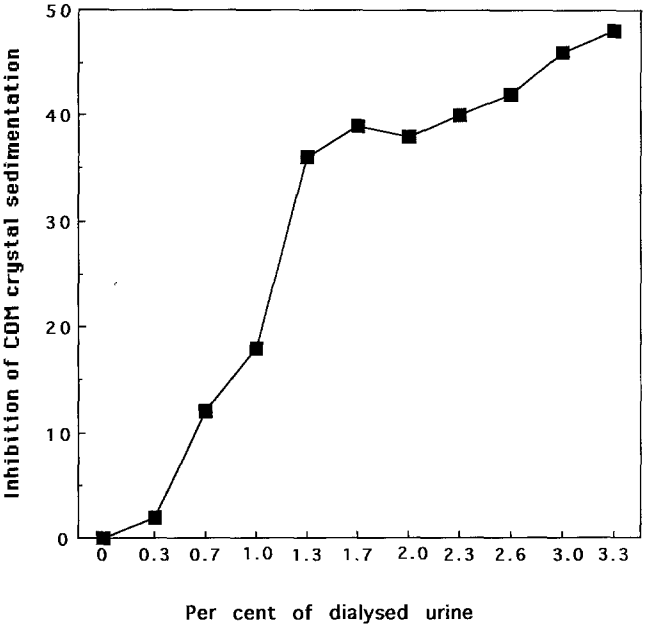

Fig. 2. Percentage inhibition of COM crystal sedimentation (crystal aggregation) at different concentrations of dialysed urine in the system. The inhibition was calculated by comparing the absorbance in the suspension containing urine with that in a reference suspension containing 0.15 mol/l sodium chloride. The crystal suspension contained 3 mg/ml COM crystals and had a pH of 7.2

that 56% of the recorded particles had a diamteter in the range 2.8–4.5 μm, 22% measured 4.6–5.4 μm and 22% 5.5–14 μm. There was a very good accordance between repeated measurements. Analysis of crystal size distribution in a Coulter counter immediately after the final recording in the spectrophotometer at 300 s showed that increased concentrations of citrate changed the distribution towards smaller crystals, despite the fact that measurements in the Coulter counter necessitated dilution of the crystal suspension. Whereas the fraction of crystals in the size range 2.8–4.5 μm was 54% with a citrate concentration of 0.3 mmol/l in the crystal suspension, the corresponding figure was 60% for a suspension containing 3.3 mmol/l of citrate. For crystals in the size range 4.6–15 μm the corresponding figures were 46% and 40% respectively.

When mixtures of citrate and dialysed urine were added to the analytical system we observed that the sedimentation rate was lower than that recorded in suspensions containing only dialysed urine, but lower than in suspension without dialysed urine or citrate. We therefore examined the sedimentation rate in systems containing dialysed urine in the presence of an extended range of concentrations down to 0.03 mmol/l. A maximal rate of sedimentation was thereby observed in the citrate interval 0.3–1.7 mmol/l, whereas lower sedimentation rates (higher inhibition) were recorded for both lower and higher citrate concentrations. These findings might indicate a competitive binding of urinary macromolecules and citrate to the same sites on the COM crystals.

The mean (SD) inhibition of crystal sedimentation measured in whole urine from NM, NW, SFM and SWF were 38.0 (12.3), 32.1 (7.5), 32.7 (12.6) and 34.7 (11.2) %, respectively. The corresponding figures for dialysed urine were 35.9 (9.8), 37.6 (8.7), 41.7 (14.7) and 38.0 (12.9) %. A significant difference in terms of inhibition of crystal sedimentation was recorded between whole urine and dialysed urine from SFM ($P<0.05$), with the higher level in dialysed urine. Higher values of sedimentation inhibition in dialysed urine were recorded in 27 of 35 (77%) SFM and in 6 of 11 (55%) SFW. Similarly 10 of 14 NM (71%) and 5 of 13 NW (38%) had a higher inhibition in the dialysed than in the whole urine. When all 73 whole and dialysed urine samples were compared the mean (SD) inhibition in dialysed urine [39.3 (12.7)] was significantly higher ($P<0.05$) than that in whole urine [33.9 (11.5)].

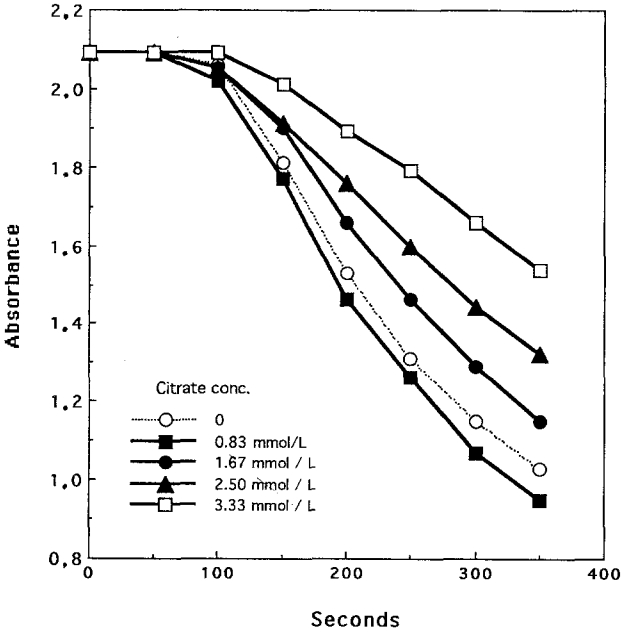

Fig. 3. The reduction in optical density in a suspension of 3 mg/ml COM crystals without and with different concentrations of citrate. The crystal suspension had a pH of 7.2. Citrate concentration: ···○··· 0; —■— 0.83 mmol/l; —●— 1.67 mmol/l; —▲— 2.50 mmol/l; –□– 3.33 mmol/l

Discussion

A high concentration of calcium oxalate crystals was used in our crystal suspension. A concentration of 3 mg/ml corresponds to an oxalate concentration of more than 20 mmol/l, which is far above that anticipated under physiological conditions. There are, however, two experimental reasons for this high crystal density. A short distance between crystals will greatly increase the propensity of aggregation and when suspensions with different concentrations of crystals were compared, we recorded the best reproducibility with suspensions containing 3 mg/l of COM.

In view of this high crystal concentration it is even more interesting that low concentrations of dialysed urine had pronounced effects on the rate of crystal sedimentation, probably explained by the aggregation inhibiting properties of urinary macromolecules [14, 15]. This indicates that marcomolecules, at least when obtained from bladder urine, are capable of counteracting crystal aggregation in an efficient way.

If an insufficient inhibition of crystal aggregation accomplished by urinary macromolecules plays a major role in the formation of stones, it is reasonable to assume that the defective control is somewhere in the nephron. At that level the concentration of macromolecular inhibitors is probably much lower than that in bladder urine. The experimental results show that although there was a very high concentration of crystals in the analytical system, a pronounced effect on the sedimentation rate was recorded even with low concentrations of dialysed urine. This gives support to the validity of using an experimental system of this type, because with these high crystal concentrations and the relatively low urine concentrations, it is less likely that the inhibitory properties are underestimated.

The demonstrated effect of citrate is of great interest because of the fact that a different result has been reported concerning its effect on crystal aggregation. Kok and coworkers [12] concluded indirectly from their crystal growth experiments that citrate was a very potent inhibitor of COM crystal aggregation, whereas Hess and coworkers [14], who used a method very similar to ours, were unable to demonstrate any effect of citrate on crystal aggregation. The different results between our experiments and those of Hess are not completely explained, but might be attributable to differences in citrate concen-

trations, crystal density and duration of the preincubation period [16]. When we omitted the preincubation, we were unable to record any effects of citrate on the sedimentation rate. If the crystals that form in the renal collecting system are retained, preincubation is certainly a reality even under physiological conditions, and our results therefore give support to the view that physiological concentrations of citrate might play an important role in moderating the initial steps of calcium stone formation.

Despite the fact that the concentration of COM crystals in our system was far above that seen physiologically, the results show that physiological concentrations of citrate counteract the aggregation of these crystals. This should be considered in view of the other important effects that citrate has on calcium oxalate crystallization [16].

The effects on the retardation of crystal sedimentation when mixtures of citrate and dialysed urine were added to the system gave some indications that citrate and macromolecules might bind to the same sites on the COM crystals, but further studies in this respect are necessary before any conclusions can be drawn. Whether the higher inhibition of crystal sedimentation in dialysed urine, compared with that in whole urine, reflects a reduction of the macromolecular inhibitory activity cannot be concluded from our measurements, because no data are available on the urine composition in these samples. There are, however, obviously factors in whole urine that can result in an aggregation that is more pronounced than in dialysed urine. Although citrate can clearly counteract such an effect, the presence of calcium or other small molecules might interfere with the electrical double layer of the crystals and result in increased aggregation.

It was shown previously that large crystal aggregates sediment more rapidly than small crystals [14], and it is therefore likely that the reduced rate of sedimentation genuinely reflects an inhibition of crystal aggregation. It is, however, difficult to prove this because measurement of crystal size distribution in a Coulter counter is not possible unless the suspension is diluted and this radically alters the aggregation properties of the suspension. It was furthermore impossible to cover the size range below 2.8 μm. Nevertheless increased concentrations of citrate appeared to result in a shift towards smaller crystals or crystal aggregates.

No attempts were made to identify the nature of those macromolecules that accounted for the inhibition of crystal aggregation in dialysed urine. Previous studies have shown, however, that both nephrocalcin [12] and Tamm-Horsfall mucoprotein [13] are efficient modifiers of calcium oxalate crystal aggregation.

In conclusion, this series of experiments showed that citrate, dialysed urine and whole urine all retarded the sedimentation rate of COM crystals in a suspension. An interaction between citrate and macromolecules might be clinically important, but further studies are necessary to verify such an effect. It will also be of great interest to assess the crystal aggregation inhibiting effect at physiological conditions, corresponding to those in the nephron. This requires analytical systems with a much lower concentration of COM crystals in the suspension and such experimental work is in progress in our laboratory.

Acknowledgements. This study was supported by grants from the Swedish Medical Research Council (project no. B91-03X-09043-02B), The County Council of Östergötland, Maud and Birger Gustavsson's Research Fund and Dr. Madaus & Co.

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