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Original Article

# *IN VITRO* EFFECT OF ACETYLSALICYLIC ACID ON CALCIUM OXALATE CRYSTALLIZATION: AN APPROACH TO ANTILITHIASIS

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**ABSTRACT**

**Objective**: In recent years, significant progress has been made in identifying and counting physico-chemical processes involved in urinary stone formation. The ability of urine to inhibit calcium oxalate crystallization is considered an important mechanism against stone formation. Several natural substances were tested to inhibit calcium oxalate crystallization. In the present study, we evaluate the effects of acetylsalicylic acid, also known as Aspirin, as an inhibitor of calcium oxalate crystallization in vitro.

**Methods:** The nucleation and aggregation of calcium oxalate crystals were studied using turbidimetric 400-sec time course measurements of optic density at 620 nm after mixing solutions containing calcium chloride and sodium oxalate at room temperature, pH 5.7. The formation of crystals is induced by the addition of the oxalate to calcium solution. The effects on calcium oxalate crystal growth of acetylsalicylic acid with various concentrations were examined. The maximum increase of optic density in the course of time reflects maximum rate of formation of new particles. After reaching equilibrium, a progressive decrease of optic density with time is observed. Rate of aggregation is derived from the maximum decrease in optic density.

**Results:** The results showed that if a concentration of acetylsalicylic acid **is** more than 1,66 mM both rate of formations of new particles and Rate of aggregation decreased ($P<0.05$).

**Conclusion:** acetylsalicylic acid has a significant effect on nucleation as well as on crystal growth stage; consequently, it inhibits the crystal formation of calcium oxalate urinary lithiasis.

**Keywords**: Urolithiasis, Calcium oxalate, Crystallization, Acetylsalicylic acid, Inhibition.

## INTRODUCTION

Urolithiasis is characterized by the formation of a stone in the kidneys or urinary tracts. A significant number of people all over the world (4-20%) are affected by urinary stone disease [1]. In addition, the recurrence rate is increases and exceeds more than 50% after **each** 10 years. The crystals of calcium oxalate (CaOx) are the primary constituents of more than 60% of the majority of human kidney stones**,** they exist in the form of CaOx monohydrate and CaOx dihydrate [2, 3]**.**

The pathogenesis of calcium oxalate stone formation is a multi-step process**.** It essentially includes: nucleation, crystal growth, crystal aggregation and crystal retention [4]. However, it was shown that the urines of normal people and stone-formers have a similar level of supersaturation [5]. Moreover, many recurrent stone-formers with no detectable metabolic abnormality show deficiencies in their urine of the naturally occurring inhibitors of crystallization [6]. The urine contains many molecules that are able to inhibit crystallization.

In spite of substantial progress in the pathophysiology and treatment of urolithiasis, there is no satisfactory drug to use in clinical therapy [7]. Endoscopic stone removal and extracorporeal shock wave lithotripsy are prohibitively costly and the recurrence is quite common [7, 8]. Thus a drug for the prevention of this disease or its recurrence would be of great interest [9].

Many plants, with complex constituents, have been studied as inhibitors in the formation of nephrolithiasis. Since there is scarcity of data concerning the potential toxic effect of these plants, we opted to study chemical drags whose effect are mastered and determined. For this reason we studied the effect of acetylsalicylic acid (ASA), named as Aspirin, containing both a carboxylic acid and an ester functional groups that may complexe calcium ions, on the inhibition in vitro of CaOx crystal. In this work, we have used the turbidimetric model [10].

## MATERIALS AND METHODS

The calcium oxalate crystallization was obtained by mixture of both calcium chloride ($CaCl_2$, $2H_2O$) 4 mM and sodium oxalate ($Na_2C_2O_4$) 10 mM solutions, containing sodium chloride (NaCl) 150 mM in order to maintain the ionic strength. These concentrations were chosen because they are close to physiological urinary concentrations. pH value was adjusted to 5.7. A pH of 5.7 was selected because it is a pH value frequently observed in the first morning urines of calcium stone formers [6]. The crystallization of calcium oxalate and the effect of the inhibitors on the crystallization kinetics were studied by turbidimetry at 620 nm, in aqueous solution with the help of a Perkin Elmer lambda 12 computer-controlled spectrophotometer [10]. Solutions, in the cuvette, were constantly stirred at 500 rpm (using a Teflon-covered stirring bar, 8 mm x 2 mm).

The different kinetic parameters of the reaction were determined on the turbidimetrical curve. The maximum increase of optic density in the course of time, called slope of nucleation (SN), mainly reflects maximum rate of formation of new particles and thus crystal nucleation. Maximum time, namely $t_{max}$, corresponds to the time between the addition of oxalate and the moment at which maximum absorbance (equilibrium) is measurable. After equilibrium has been reached, crystals could neither nucleate nor grow. A progressive decrease of optic density in the course of time was observed. The maximum slope of decrease of optic density at 620 nm in the course of time therefore represented the rate of decrease of the particle amount due to crystal aggregation. As optic density decrease, crystal aggregation increase. Rate of aggregation, was derived from the maximum decrease in optic density called slope of aggregation (SA).

For similar conditions, the experiment was repeated six times. As such, the main, standard deviation (SD) and variation coefficients (VCs) were calculated. All VCs were less than 6.6 %, suggesting that under the same experimental conditions, an identical distribution of particle number and size was produced.

**Crystallization without inhibitor**

A volume of 2 ml of the calcium chloride solution of 4 mM was transferred into a measurement cuvette having 1 cm of optical path, to which we added 1 ml of sodium oxalate of 10 mM. Then, measurement was immediately performed. The final concentrations in this test were 2.67 and 3.34 mM, respectively. All the chemical products utilized were Panreac products (Spain) which have analytical purity. The kinetic curve obtained by turbidimetry representing the three crystallization phases (nucleation, growth, and agglomeration) is shown in Fig.1.

**Crystallization with inhibitor**

The inhibitor effect on the different crystallization phases was studied in the same experimental conditions, as above. Nevertheless, it is noteworthy to mention that the inhibition substance was added to the calcium solution before mixing it to calcium chloride. We tested acetylsalicylic acid (Rhodia, french) at final concentrations of 0.834 mM, 1.66 mM, 3.34 mM and 5 mM.

**Statistics**

Data were expressed as mean values ± SD. The Student tests, for paired comparisons within groups, were used. Percentage inhibition was calculated as [1- (SNa / SNc)] x100 for rate of nucleation and [1- (SAa / SAc)] x100 for the rate of aggregation [10-12], where (a) stands for acetylsalicylic acid as inhibitor and (c) for control. P<0.05 was considered significant. Acetylsalicylic acid inhibitor effect on calcium oxalate crystallization was controlled against pure calcium oxalate crystallization.

**RESULTS**

Time-course measurements of optic density at 620 nm under standard conditions (4 mM calcium, 10 mM oxalate) were illustrated in fig. 1(a).

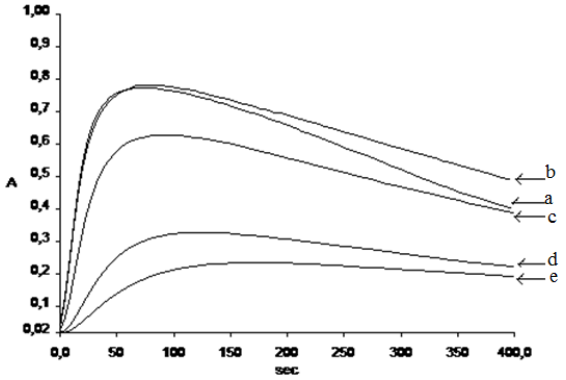


**Fig. 1: average of change in turbidity without and with acetylsalicylic acid (inhibitor) (a): Control, (b): ASA at 0.83 mM, (c): ASA at 1.66 mM, (d): ASA at 3.34 mM, (e): ASA at 5 mM**

The amount of crystals formed increased in the course of time until a maximum was reached. After reaching equilibrium at 74 sec, optic density progressively fell due to crystal aggregation. The inhibitory effect of acetylsalicylic acid on the nucleation of calcium oxalate crystal is shown in table 1 and Fig.1 (b, c, d, and e). In the presence of Acetylsalicylic Acid at the concentration lower than 0.834 mM, $t_{max}$ and the slope of nucleation were not influenced (p> 0.05), only the slope of aggregation decreased (P = 0.002).

But when the concentration of Acetylsalicylic Acid was more than 1.66 mM, $t_{max}$ increased and the slopes of calcium oxalate crystal growths (SN and SA) decreased (P<0.05).

**Table 1: Effects of acetylsalicylic acid concentration on calcium oxalate crystallization**

| Concentration of ASA | 0 mM (control) | 0.834 mM | 1.66 mM | 3.34 mM | 5 mM |
|---|---|---|---|---|---|
| Tmax (sec) | 74 ± 7.48 | 74 ± 7.04 | 94 ± 9.71 | 130 ± 7.48 | 184 ± 17.52 |
| | | P = 0.101 | P = 0.004 | P < 0.001 | P < 0.001 |
| SN ($x10^{-3}$) | 24.68± 1.11 | 24,33 ± 1,03 | 17,1 ± 2.07 | 5.4± 0.38 | 0,64 ± 0.05 |
| | | P = 0.568 | P < 0.001 | P = 0.001 | P < 0.001 |
| SA ($x10^{-3}$) | 1.22 ±0.091 | 1,01 ±0.071 | 0, 84 ± 0.05 | 0.44 ±0.04 | 0.22 ±0.027 |
| | | P = 0.007 | P < 0.001 | P < 0.001 | P < 0.001 |

At the highest concentration of acetylsalicylic acid, nucleation inhibition ratio was 97.42% and crystal aggregation ratio was inhibited by approximately 81.57% (table 2).

**Table 2: Evolution of the inhibition rate, according to acetylsalicylic acid concentration**

| Concentration of ASA (mM) | 0.834 | 1.66 | 3.34 | 5 |
|---|---|---|---|---|
| - Rate of [ASA/Calcium] | 0.31 | 0.62 | 1.25 | 1.87 |
| - Rate of nucleation inhibition (%) (RN) | 1.42 | 30.48 | 78.13 | 97.42 |
| - Rate of aggregation inhibition (%) (RA) | 17.21 | 31.28 | 63.66 | 81.57 |

The reproducibility of the measurements was good [coefficients of variation (CVs) % < 6.6%], suggesting that under the same experimental conditions, an identical distribution of particle number and size was reproducible.

**DISCUSSION**

It is well known that the supersaturation of urine is responsible for calcium oxalate stone formation [1]. In addition, when urinary supersaturation with calcium oxalate exceeds the limit of metastability, the nucleation of microcrystals occurs. Two separate processes lead to urinary stones, namely nucleation and aggregation of calcium oxalate crystals [12]. In the present study, we have followed the formation of calcium oxalate crystals in a spectrophotometer cuvette by the light scattered after mixing solutions of calcium chloride and sodium oxalate (fig.1). The maximum increase in optic density at 620 nm in the course of time was related essentially to crystal growth [6]. Optic density is an exact measurement of particle concentration per unit volume [3]. Another kinetic parameter, the maximum time ($t_{max}$), relied to the time between the addition of oxalate and the moment at which maximum absorbance (equilibrium) is measurable. Any prolongation in the beginning of nucleation will affect the time that $t_{max}$ is reached [10].

With high concentrations of calcium and oxalate, the supersaturation exceeds the metastability [6, 10]. Consequently, prevention of renal stone requires low urinary concentrations of calcium and oxalate. However, concentrations of calcium and oxalate are not different in the urines of many stone-formers. The difference is that the excretion of inhibitor substances**,** which complex calcium or oxalate**,** is diminished in the urines of stone-formers [13].

Inhibitors of calcium stone formation prevent crystals growth and their aggregation in three ways. First, by coating the surface of growing crystals. Second, by complexing the calcium, such as various physiological inhibitors of urolithiasis found in urine including

inorganic [9] (eg. Magnesium) and organic (eg. Citrate). Finally, by facilitating their elimination (Urinary Prothrombin Fragment1, glycosaminoglycans) [14, 15]. Those substances are known to inhibit stone formation.

The present study showed that the acetylsalicylic acid at lower concentration (less than 0.83 mM) had a positive effect on the aggregation of the crystals because the slope of aggregation decreases (P = 0.007). Acetylsalicylic acid adsorbed by the surface of the crystal, thereby inhibit**s** crystal aggregation [5]**,** but it did not alter $t_{max}$ and the rate of nucleation significantly (P > 0.05).

When the concentration of acetylsalicylic acid is more than 1.66 mM, a significant inhibition effect of acetylsalicylic acid is observed**.** The later increased $t_{max}$ and also reduced strongly the rates of nucleation and aggregation (P<0.05), due to the significatively decreasing of the slopes of calcium oxalate crystal's growth (SN and SA). As higher carboxylic acid, acetylsalicylic acid chelates calcium and form soluble salt which is excreted through urine [16].

The inhibition rate of crystallization varies when the concentration ratio of [ASA/Calcium] increases (table 2). Moreover, the inhibition rate of aggregation increases by increasing the concentration of ASA or the concentration ratio [ASA/calcium]. At the highest concentration of acetylsalicylic acid, the nucleation percentage inhibition ratio was 97.42% and the crystal aggregation was inhibited by approximately 81.57%.

The interference with crystal growth and aggregation therefore seems a possible therapeutic strategy for the prevention of recurrent stone disease [17]. The ability of acetylsalicylic acid to reduce the nucleation increases the metastable limit of oxalate in urine and prevents the precipitation of the CaOx crystal. Acetylsalicylic Acid produces its action similarly to the natural urinary inhibitors, and inhibits crystals aggregation and growth. It was found to be the most potent inhibitor of calcium oxalate crystals.

## CONCLUSION

This work showed that the acetylsalicylic acid can be an interesting inhibitor of urinary stones formation of calcium oxalates, which is the most common promoter of urinary lithiasis. Further new studies in vivo should be done to assess the effect on calcium oxalate crystallization.

## CONFLICT OF INTERESTS

Declared None

## REFERENCES

1. Strope SA, Wolf JS, Hollenbeck BK. Changes in gender distribution of urinary stone disease. Urol 2010;75(3):543-6.
2. Benramdane L, Bouatia M, Idrissi MOB, Draoui M. Infrared analysis of urinary stones, using a single reflection accessory and KBr pallet transmission. Spectrosc Lett 2008;41:72-80.
3. Bensatal A, Ouahrani MR. Inhibition of crystallization of calcium oxalate by the extraction of Tamarix gallica L. Urol Res 2008;36:283-7.
4. Kavanagh JP. *In vitro* calcium oxalate crystallisation methods. Urol Res 2006;34:139-45.
5. Chauhan CK, Joshi MJ. Growth inhibition of Struvite crystals in the presence of juice of Citrus medica Linn. Urol Res 2008;36:265-73.
6. Kulaksizoğlu S, Sofikerim M, Cevik C. *In vitro* effect of lemon and orange juices on calcium oxalate crystallization. Int Urol Nephrol 2008;40:589-94.
7. Spivacow FR, Negri AL, Polonsky A, Del Valle EE. Long-term treatment of renal lithiasis with potassium citrate. Urol 2010;76(6):1346-9.
8. Prasad KVSRG, Sujatha D, Bharti K. Herbal drugs in urolithiasis: a review. Pharmacog Rev 2007;1(1):175-8.
9. Basavaraj DR, Biyani CS, Browning AJ, Cartledge JJ. The Role of urinary kidney stone inhibitors and promoters in the pathogenesis of calcium containing renal stones. EAU-EBU 2007;5:126-36.
10. Driouch A, Djelloul A, Kaid-Omar Z, Semmoud A, Rais A, Addou A. Optimized experimental design for the inhibition of calcium oxalate using a turbidimetrical model. Asia-Pac J Chem Eng 2008;3:425-31.
11. Prachi K, Vinod KM, kakkar A, Neetu B, Rajendra S. Study on *in vitro* anti-lithiatic activity of Phyllanthus niruri linn. Leaves by homogenous precipitation and turbiditory method. Int J Pharm Pharm Sci 2014;6(4):124-7.
12. Surendra KP, Kartik C, Ranjit H. *In-vitro* calcium oxalate crystallization inhibition by Achyranthes indica linn. Hydroalcoholic extract: An approach to antilithiasis. Int J Pharm Bio Sci 2011;2(1):432-7.
13. Stoller ML, Chi T, Eisner BH, Shami G, Gentle DL. Changes in urinary stone risk factors in hypocitraturic calcium oxalate stone formers treated with dietary sodium supplementation. J Urol 2009;181(3):1140-4.
14. Singh SK, Aggarwal KP, Tandon S, Tandon C. Proteomic analysis of human calcium oxalate renal stone by 2-D PAGE and in vitro analysis of bioactivity of the proteins. EAU 2012;11(1, Suppl 1):855.
15. Aggarwal KP, Tandon S, Naik PK, Singh SK, Tandon C. Novel antilithiatic cationic proteins from human calcium oxalate renal stone matrix identified by MALDI-TOF-MS endowed with cytoprotective potential: an insight into the molecular mechanism of urolithiasis. Clin Chim Acta 2013;415(16):181-90.
16. Emel A, Mualla O. inhibition of calcium oxalate monohydrate crystal growth using polyelectrolytes. J Cryst Growth 2007;307:137-44.
17. Atmani F, Sadki C, Aziz M, Mimouni M, Hacht B. Cynodon dactylon extract as a preventive and curative agent in experimentally induced nephrolithiasis. Urol Res 2009;37(2):75-82.