**INTRODUCTION**

Urolithiasis is characterized by the formation of a stone in the kidneys or urinary tract. 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 of great interest [9].

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 complex 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 (CaCl₂, 2H₂O) 4 mM and sodium oxalate (Na₂C₂O₄) 10 mM solutions, containing sodium chloride (NaCl) 150 mM in order to maintain the ionic strength. These concentrations were 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].

The different kinetic parameters of the reaction were determined on the turbidimetric curve. The maximum increase of optic density in the course of time reflects maximum rate of formation of new particles and 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- (SA / 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).

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, tmax 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, tmax 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

<table>
<thead>
<tr>
<th>Concentration of ASA (mM)</th>
<th>0 mM (control)</th>
<th>0.834 mM</th>
<th>1.66 mM</th>
<th>3.34 mM</th>
<th>5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmax (sec)</td>
<td>74 ± 7.48</td>
<td>74 ± 7.04</td>
<td>94 ± 9.71</td>
<td>130 ± 7.48</td>
<td>184 ± 17.52</td>
</tr>
<tr>
<td>SN (x10^-3)</td>
<td>24.68 ± 1.11</td>
<td>24.33 ± 1.03</td>
<td>17.1 ± 2.07</td>
<td>5.4 ± 0.38</td>
<td>0.64 ± 0.05</td>
</tr>
<tr>
<td>SA (x10^-3)</td>
<td>1.22 ± 0.091</td>
<td>1.01 ± 0.071</td>
<td>0.84 ± 0.05</td>
<td>0.44 ± 0.04</td>
<td>0.22 ± 0.027</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Concentration of ASA (mM)</th>
<th>0.834</th>
<th>1.66</th>
<th>3.34</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of ASA/Calcium (%)</td>
<td>0.31</td>
<td>0.62</td>
<td>1.25</td>
<td>1.87</td>
</tr>
<tr>
<td>Rate of nucleation inhibition (%) (RN)</td>
<td>1.42</td>
<td>30.48</td>
<td>78.13</td>
<td>97.42</td>
</tr>
<tr>
<td>Rate of aggregation inhibition (%) (RA)</td>
<td>17.21</td>
<td>31.28</td>
<td>63.66</td>
<td>81.57</td>
</tr>
</tbody>
</table>

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 (tmax), 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 tmax 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 Fragment I, 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 absorbed by the surface of the crystal, thereby inhibits 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 significantly 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