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In vitro Growth Inhibition Study of Urinary Type Brushite Crystals in the Presence of Healthy Adult Urine and Tartaric acid

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Abstract: *Brushite or calcium hydrogen phosphate dehydrate is commonly found in urinary stones. In the present study the single diffusion gel growth technique was used to grow brushite crystals in silica hydro gel medium. It provides controlled environment to simulate the growth of brushite containing urolithiasis under static conditions in the presence of healthy human urine. In this in vitro study, the supernatant solution containing calcium chloride was used to grow brushite crystals and considered as control. To study the effect of urine on the growth of brushite crystals, different volume of healthy human urine was added in the supernatant solution. In this in vitro study, the role of healthy human urine on the growth of brushite crystals in terms of effect of the pH of supernatant solutions and volume of urine on the morphology, size and number of grown crystals were studied. The inhibition effect of tartaric acid was verified by adding tartaric acid to urine and calcium chloride containing supernatant solution. Tartaric acid was selected because tamarind contains tartaric acid and quite frequently it is used in food preparations. Tartaric acid as well as natural urine both was found to be good inhibitors of brushite crystal growth. The addition of tartaric acid along with urine in the supernatant solution stopped the nucleation of brushite crystals. Results are discussed.*

Keywords: *Bio-crystallization, Biomaterials, Crystal morphology, Calcium compounds, Growth from solution, Growth inhibition*

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I. Introduction

Calcium salt stones are most common urinary calculi, which may be pure calcium oxalate stones or calcium oxalate and calcium phosphate mixed stones and occasionally calcium phosphate stones. Calcium phosphate types are either appetite ($Ca_{10}(PO_4)_6(OH_2)$) or brushite ($CaHPO_4\ 2H_2O$). This has attracted several workers to grow brushite, that is, calcium hydrogen phosphate dihydrate (CHPD) crystals by gel growth method and characterize them by various techniques [1-4].

Generally, the urinary calculi are composed of crystalline components. Multiple steps are involved in the pathogenesis of these urinary crystals, which are nucleation, growth and aggregation. The stone formation *in vivo* requires supersaturated urine and certain litho genetic conditions such as urinary pH, ionic strength, solute concentration, certain glycoprotein and complexation. In the present study, the single diffusion gel growth technique is considered as a simple model for urinary crystals or urinary calculi growth, however, the growth of urinary calculi is much more complex in human body and under the dynamic condition due to continuous flow of urine and change in the concentration of solutes. Notwithstanding, the gel growth method using glass test tubes for the growth of millimeter size brushite crystals [2-5] and the modified gel growth technique for the growth of microscopic size brushite crystals [6,7] have been reported. The gel growth technique is recently reviewed in terms of successful journey of an in vitro model from vision to reality [8].

The gel growth based model has been successfully employed to test the growth inhibition of brushite crystals in different herbal extracts and weak acid solutions, for example, tartaric acid and tamarind [9,10], aqueous extracts of *Tribulus terrestris* and *Bergenia Ligulata* [11], citric acid [12], leaf extracts of *Chenopodium album* [13] and aqueous extract of *Arava lanata* [14].

Human urine possesses four different types of inhibitors, viz., (1) small organic anions such as citrate, (2) small inorganic anions such as pyrophosphates, (3) multivalent cations such as magnesium and (4) macromolecules such as osteopontin and Tamm-Horsfall protein [15]. Urine inhibits growth, aggregation, nucleation and cell adhesion of calcium oxalate crystals [16].

In the present investigation, the growth of brushite crystals was carried out with different volume of human urine added into the supernatant solution containing calcium chloride solution and its effect, on the average length of the crystals, the number of the grown crystals and the type of morphologies of the crystals were studies. Tartaric acid and tamarind has been proved as a good inhibitor of brushite crystals [9] and tamarind possesses tartaric acid [17] and hence in the present investigation tartaric acid and human urine both were used together to verify growth inhibition of brushite crystals.

II. Experimental Texhnique

The glass test tubes of 2.5 cm diameter and 15 cm length were used as a crystal growth apparatus. To set the gel, sodium metasilicate solution of specific gravity 1.08 was acidified by 1.5 M orthophospheric acid solution in such a manner that the desired pH value could be set for the mixture, in the present study 4.5, 5.4 and 5.6 pH values were chosen. The mixture was then poured in to respective test tubes for setting the gel. After setting the gel, different supernatant solutions containing calcium chloride were poured on the set gels. The crystals were found to be growing within two days of pouring the supernatant solutions. The growth was completed within a month. Elongated needle type and star shaped crystals were grown in the gel. Figure1 shows the types of crystals grown in the gel.

Figure 1: Brushite Crystals grown in gel media

The apparent length of growing crystals was measured by traveling microscope of 0.001 cm least count. The statistical analysis of the single factor ANOVA was applied to find correlation between two different variables in different plots. The p values were calculated. The value $p<0.05$ indicates highly correlated nature.

Human urine of healthy male volunteer was selected after routine clinical laboratory test. The following supernatant solutions were prepared by dissolving 5mg of CaCl2 in constant volume of water, pure urine as well as water and urine mixtures:

1. 00 ml Urine + 24 ml Distilled water + 5 gm $CaCl_2$
2. 06 ml Urine + 18 ml Distilled water + 5 gm $CaCl_2$
3. 12 ml Urine + 12 ml Distilled water + 5 gm $CaCl_2$
4. 18 ml Urine + 06 ml Distilled water + 5 gm $CaCl_2$
5. 24 ml Urine + 00 ml Distilled water + 5 gm $CaCl_2$

To study the effect of tartaric acid on the growth of brushite crystals, the 0.01M, 0.05M, 0.1M, 0.5M and 1.0 M solutions of tartaric acid were added in equal amount to calcium chloride in the supernatant solution and the length and number of grown crystals was noted down during the growth by using travelling microscope. This experiment was done at pH values of 4.5, 5.4 and 5.6. Moreover, to verify the effect of tartaric acid on the growth of brushite crystals, a supernatant solution containing the mixture of 8 ml of 1M calcium chloride, 8 ml of 1M tartaric acid and 8 ml of human urine was used. Figure 2(a,b) shows the photographs of CHPD crystals grown in the test tubes in the presence of 8 ml and 16 ml human urine in the supernatant solution.

2(a) 2(b)

Figure 2(a, b): CHPD crystal growth in the presence of 8 ml and 16 ml human urine in the supernatant solution

The grown crystals were characterized by thermo gravimetric analysis and powder XRD and were confirmed as CHPD crystals. Figure 3 shows the powder XRD pattern of CHPD crystal.

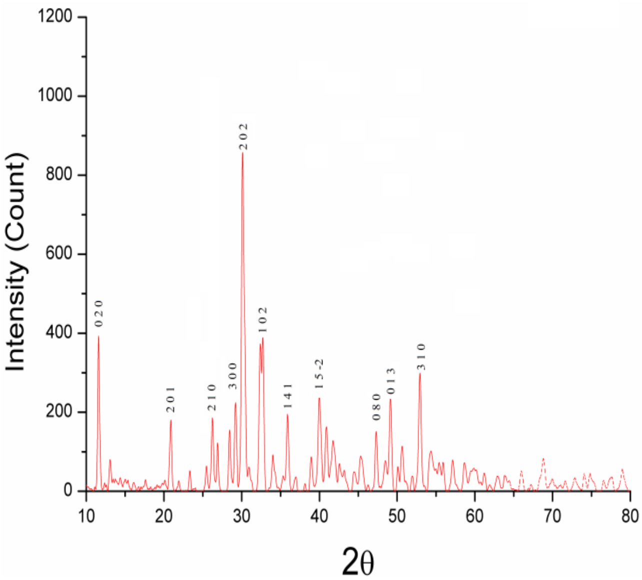

Figure 3: XRD pattern of brushite crystals

The powder XRD patterns were recorded on Philips X'pert MPD system using Cu (Kα) radiation with the step size 0.01ºC and scanning time of 1s. The XRD patterns were analyzed with the help of software Powder-X. The unit all parameters were found to be: a=5.812 Å, b = 15.180 Å, c= 6.239 Å and β= 116^0 which correspond to the reported values [18].

III. Results And Discussion

The saturation and solubility product in water are, generally, simple to define but are more complex in urine. When the concentration of a substance in urine reaches a point at which the saturation would take place in water, the crystallization does not take place as expected in urine. Altogether, urine has the ability to hold more solute in the solution than pure water. The mixtures of many electrically active ions in urine cause interaction that changes the solubility of the components. However, many organic molecules such as urea, uric acid, citrate and complex mucoproteins of urine, affect the solubility of other substances in urine, which has been discussed by Menon et al. [19]. The influence of citric acid and lemon juice on the growth of CHPD or brushite crystals in presence of human urine and artificial reference urine has been reported by Joshi and Joshi [4]. The effect of seed crystals of hydroxyapatite or brushite on the crystallization of calcium oxalate in undiluted human urine *in vitro* conditions has been reported by Grover et al. [20]. The interaction of small and macromolecules with growing CHPD crystals was reported by Sikric et al. [18] using brushitevas a good model crystal to study the aspects of interaction between additives and crystal. Moreover, the scanning probe microscopy has been employed by Giocondi et al. [21] to investigate the role of several solution parameters and additives on the brushite atomic strep motion. It has been further found by the same workers that the activation barrier for phosphate rather than calcium incorporation limits the growth kinetics and the additives, such as citrate, influence the step motion in distinctively different ways, which is helpful to provide details how and where molecules inhibit or accelerate kinetics.

The effect of human urine on the number of crystals grown and their morphology is studied in the present investigation. In general, two types of crystals morphologies of brushite crystals were observed, that is, elongated needle type and star type. In the star type morphology, different needle were coming out from the common center. The lengths of star as well as needle type crystals were measured and the numbers of star and needle type crystals growing in the test tubes were counted. Figure 4 shows the plots of average number of needle type, star type as well as needle and star type both together (or total number) crystals versus volume of human urine. The statistical analysis ANNOVA single factor analysis is performed and *p* values are given in the inset table along with the figure.

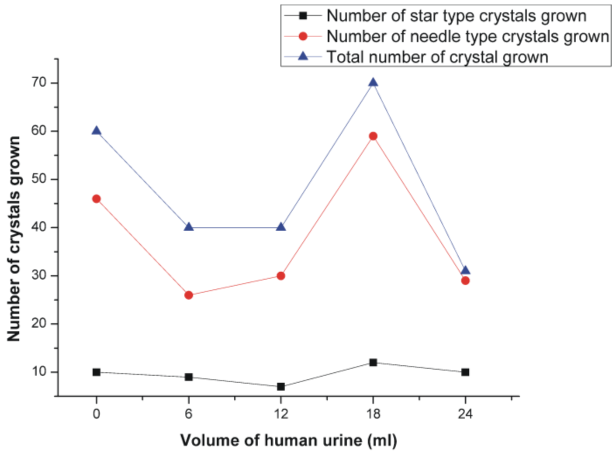

Figure 4: Number of crystals grown Vs. volume of urine

The *p* values for the number of needle type and total number of crystals grown are significant at the level of 0.002, but for the number of star type crystals grown the *p* value is very high indicating the level of 0.590 due to almost independent nature of the number of star type crystals grown from the volume of urine, i.e., the plot is almost parallel to X-axis. This shows that the number of both needle and star type crystals vary with the volume of urine with minimum number between 5 ml to 12 ml of urine volume. The effect of pH of supernatant solution on the number of needle type, star type as well as needle and star both types crystals together shows that the minimum number of crystals are grown in the pH range of 5.8 to 6.9, which is shown in figure 5.

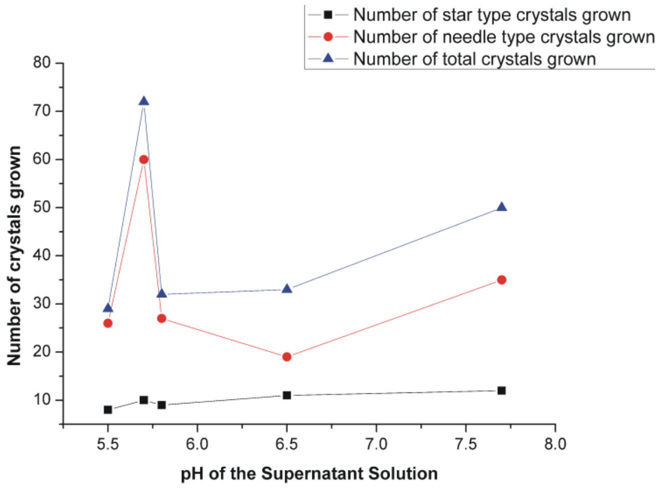

Figure 5: Number of crystals grown Vs. pH of supernatant solution

The statistical analysis ANNOVA single factor analysis results for *p* values are compiled in inset figure and found to be significant.

The freshly voided urine has a slightly acid reaction. The normal pH range is 5 to 8 and this includes abnormal pH range also [22]. Usually, three types isohydruria are described; (1) acid type having pH range 4.5-5.5 is associated with precipitation of uric acid; (2) mild acid to neutral type with pH range 5.5-6.0 is associated to the precipitation of calcium oxalate; and (3) alkaline type having pH range 7-7.5 is related to the precipitation of phosphates [23]. In the present study, the values of pH for minimum phosphate precipitation is in the range of 5.8 to 6.9, which corresponds to the range of pH values where neither phosphate nor oxalate type of calculi likely to be precipitated. However, for the narrow pH range from 5.5 to 5.8 a sharp increase in the number of grown crystals is found as that can be seen from figure 5. This also further suggests that natural urine is having inherent inhibitors; however, they do not completely stop the precipitation and growth of the crystals but minimize the precipitation and growth.

The effect of human urine on the average length of grown crystals was studied. The plot of average length of grown brushite crystals versus volume of human urine is shown in figure 6, which indicates that the average apparent length of grown crystals is the minimum at 18 ml of urine in the supernatant solution.

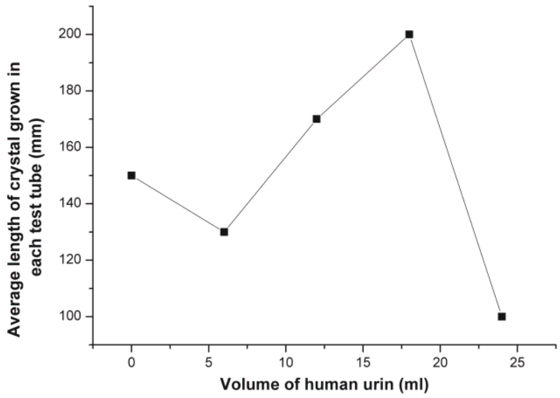

Figure 6: The plot of average length of grown brushite crystals Vs. volume of human urine.

The statistical analysis ANNOVA single factor shows the value of p of 0.087 for the average length of grown brushite crystals versus volume of human urine, which more than the level of significance. The reason may be given as the average length is considered for both star type and needle type crystals, but as per the figure 4 the star type crystals exhibit high *p* value and that may be influencing the average length resulting in to high *p* value. The effect of pH of supernatant solution on the average apparent length of grown crystals is shown in the plots of figure 7.

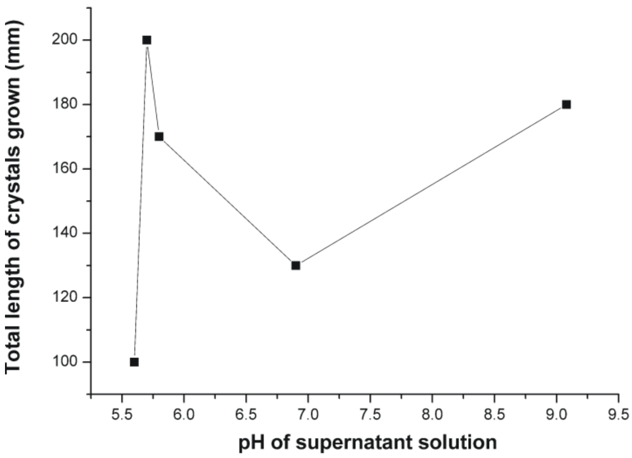

Figure 7: The average length of grown crystals Vs. pH of supernatant solution

This shows that the minimum average apparent length of brushite crystals is achieved for pH 5.7, that is, slightly acidic urine. Moreover, the minimum average apparent length value occurring at pH 5.7 is slightly lower than the minimum number of crystals grown, that is, for pH 5.8 to 6.5. However, the average length of crystals is less than 4.5 mm and it is the minimum at pH 5.7, which is nearly of 3.2 mm average length. It is important to note that this value is below the maximum length of a crystal that can be flushed out through urethra. Moreover, the single factor ANNOVA analysis suggesting that the value of *p* for the pH of supernatant solution on the average apparent length of grown crystals is significant.

The inhibition of mineralization of urinary calculi forming minerals has been demonstrated by naturally occurring acid, e.g., lactic acid, malic acid, citric acid and tartaric acid, in terms of chelating effect of the Ca^{2+} and screening from the precipitating anions like phosphates, oxalates and carbonates by Rao and Bano [24]. Tartrates are expected to form metal ion complexes with calcium, thus the presences of tartrates in urine may decrease the amount of calcium available for oxalate precipitation [25]. Tartrates bind with cations needed for crystal formation and subsequent growth and, therefore, function as crystal growth inhibitors for calcium oxalate by chemical absorption on the crystallization sites at a growing interface [26]. This was also further confirmed by Yuan and Ouyang [10] using tartaric acid and its salts.

To study the effect of tartaric acid on the growth of brushite HPD crystals, 0.01M, 0.05M and 1.0 M tartaric acid solutions were added in equal amount to calcium chloride in the supernatant solution and the length and number of grown crystals is found out. The plots of figure 8 show the relationship between the number of brushite crystals grown with the molar concentration of tartaric acid for different pH values of setting the gel, viz., 4.5, 5.4 and 5.6.

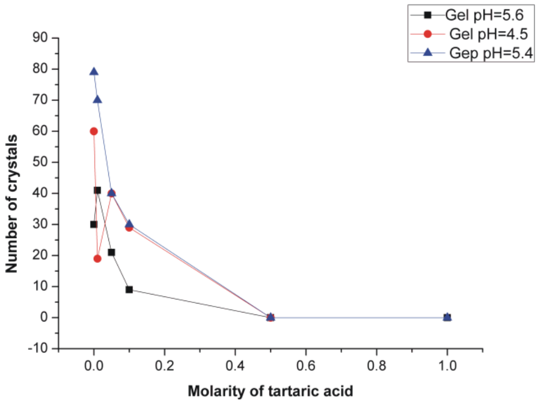

Figure 8: The number of brushite crystals grown Vs. the molar concentration of tartaric acid for different pH values

From the single factor ANNOVA analysis indicating the low p values i.e. the number of grown crystals is highly dependent on molarities of tartaric acid. It can be seen that for the pH value 5.4 the number of crystals steadily decreases. But for the Ph values of 4.5 and 5.6 the maxima and minima are observed in the plots, however, the overall nature of the plots for these values is of decreasing nature with number crystals grown at 0.5 M concentration of tartaric acid. For different pH values the gel density and pore size distribution changes. The changes in pore size distribution facilitate the movement of different ions in different manners according to the size of ions and pores available and hence the variation in the plots, in terms of maxima and minima, may be due to these shifts in the nature. Altogether, at higher concentration of tartaric acid, the inhibition is in complete form and no crystals are grown at 0.5 M concentration of tartaric acid. The effect of molarity of tartaric acid on the average length of grown brushite crystal is studied.

The effect of tartaric acid on the growth of brushite crystals was tested by adding 12 ml $CaCl_2$ solution and 12 ml tartaric acid solution as a supernatant solution. Only needle type crystals were grown and their average number was 25 crystals. However, the addition of natural urine into tartaric acids and calcium chloride solution gave interesting results. By adding 8 ml natural urine to 8 ml $CaCl_2$ solution and 8 ml tartaric acid solution, it was observed that the number of grown crystals decreased to nil. This proves that the increased inhibition due to the presence of tartaric acid and urine. Urine and tartaric acid were found to be good inhibitors of brushite growth *in vitro* conditions. This study may help to device the proper technique for medical management of brushite type crystals by varying the pH of urine. This may also lead to reverse pharmacology, i.e., a trans-discipline initiating the drug discovery and development from traditional knowledge and objective experiential documentation [27].

IV. Conclusion

The number of grown brushite crystals varied with urine volume and pH of the supernatant solution in the gel growth study. The minimum number of crystals was grown at 12 ml of urine solution, and pH range 5.8 to 6.9. The average length of brushite crystals was the minimum for 18 ml urine and at pH 5.7. The addition of tartaric acid and urine into the supernatant solution of calcium chloride stopped the nucleation of the brushite crystals by chelating tartaric acid with calcium ions. Natural urine shows inhibition of brushite crystals in the pH 5.8 to 6.9, which is within the urine pH in human body.

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