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RESEARCH ARTICLE

EFFECT OF URINE SAMPLES OF EXPERIMENTAL RATS ON THE GROWTH OF CALCIUM OXALATE CRYSTALS *IN-VITRO*

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ABSTRACT

Urinary calculi formation is a very complex phenomenon taking place in a step-wise manner leading to urinary stone disease. Formation of urinary crystals is an essential step in stone disease. Common known stone components are calcium oxalate, calcium phosphate and uric acid. *In- vitro* studies was done by growing crystals in silica gel medium. Urine samples of the non-diabetic non-calculogenic, diabetic non-calculogenic, non-diabetic sodium oxalate induced calculogenic, non-diabetic ethylene glycol induced calculogenic, diabetic sodium oxalate induced calculogenic and diabetic ethylene glycol induced calculogenic were collected and added in requisite amount on the top of the gel. Length of the crystal column and size of the crystals were noted on days 1, 7, 14, 21 and 30 respectively. Comparative study of the mean length of crystal column *in- vitro* showed the maximum crystal growth in non-diabetic ethylene glycol induced calculogenic rat urine added set. Comparative study of mean length of crystal column between the diabetic non-calculogenic and diabetic calculogenic showed maximum crystal growth in the diabetic calculogenic rat urine added set. Analysis of Variance showed statistically significant difference in mean values among the groups and the size of the calcium oxalate crystal *in- vitro* showed significant difference in mean ($p=0.001$). Highest mean of $398.75 \pm 1.25\mu$ was seen in the diabetic sodium oxalate induced calculogenic rats. Duncan's Multiple Range Test showed maximum crystal size in the diabetic sodium oxalate induced calculogenic rats compared to other groups. *In- vitro* growth of calcium oxalate crystals was maximum in the diabetic urine added set. This indicates the tendency of diabetes along with calculogenesis for promoting maximum crystal growth.

INTRODUCTION

Urinary stone disease has now become wide spread and is posing a significant health problem. Crystalluria is one of the risk factors for the development of urolithiasis (**Tiselius, 1997**). The incidence of urinary calculi is increasing worldwide and calcium oxalate is the predominant component of most stones, followed by struvite, cystine, uric acid and other compounds (**Trincheri, 2006**). Calcium oxalate monohydrate, the thermodynamically most stable form, more common in clinical stones than calcium oxalate dihydrate, has a greater affinity for renal tubular cells, and is thus responsible for the formation of stones in the kidney (**Verkoelen *et al.*, 1995; Sarmistha Saha and Ramtej J.Verma, 2013**). The crystallisation of the calcium oxalate begins with increased urinary supersaturation, with the subsequent formation of the solid crystalline particles within the urinary tract. This is followed by nucleation, by which stone-forming salts in supersaturated urinary solution coalesce into clusters, that then increase in size by the addition of new constituents (**Basavaraj *et al.*, 2007**).

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It is understood that crystal adhesion encourages stone pathogenesis but normally this is prevented due to the presence of a number of naturally occurring macromolecules in the urine that inhibit the attachment of crystals to the renal cells. Studies have shown that even though some of these natural macromolecules have a protective response to crystal attachment and prevent stone formation, at times they may react to inflammation induced and thus some of them are promoters and not inhibitors (**Rosemary Lyons Ryall, 2011**). Diabetes mellitus has a deleterious effect on multiple organ systems, particularly the urinary tract (**Dornfield, 1986**). An increased prevalence of nephrolithiasis in patients with diabetes was observed as compared with patients without diabetes (**Meydan *et al.*, 2003**). The stone formation in our body is similar to the crystal growth (**Werness *et al.*, 1981; Khan *et al.*, 1993**) and can be grown synthetically. The two known forms of calcium oxalate are calcium oxalate monohydrate (whewellite) and calcium oxalate dihydrate (weddelite). The aim was to study the effect of the urine samples of non-diabetic non-calculogenic, diabetic non-calculogenic, non-diabetic sodium oxalate induced calculogenic, non-diabetic ethylene glycol

induced calculogenic, diabetic sodium oxalate induced calculogenic and diabetic ethylene glycol induced calculogenic on the growth of calcium oxalate crystals *in-vitro*.

MATERIALS AND METHODS

The experimental work was done in male rats of wistar species each weighing 200-250 gm. Diabetes was induced in the experimental group of rats by injecting 3% aqueous solution of alloxan monohydrate in a dose of 150 mg/kg body weight. Calculogenesis was induced in rats by giving orally 0.5% ethylene glycol and 0.1% sodium oxalate respectively. Six rats were included in the experimental group and six in the control group and the experiment was conducted for a period of three months. Urine samples were collected separately for the study.

The calcium oxalate crystals whewellite and weddellite were grown in-vitro in silica gel medium by single diffusion method. 20 ml of sodium metasilicate solution of density 1.03g/cm^3 was taken and the pH was adjusted to 6 using 3M acetic acid. To this, 5ml of 1M calcium chloride was added. The solution was mixed well and set aside overnight for gel formation. Two sets, each consisting of five tubes with gel were arranged for each experiment. Next day, 5ml of 1M oxalic acid was added on top of the gel. In one set, 5ml of urine of the control group and in the second set, 5ml each of the urine samples of the experimental groups were added to the top of the gel. Crystals appeared as a cloudy precipitate. Thickness (length) of crystal column and size of crystals were noted on days 1, 7, 14, 21 and 30 respectively. Size of the crystals was measured using a micrometer and thickness of crystal column by Vernier calipers. Statistical tests Analysis of Variance and Duncan's Multiple Range Test was done.

RESULTS AND DISCUSSION

Comparative study of mean length of the crystal column showed maximum crystal growth in the non-diabetic ethylene glycol induced calculogenic rat urine added set.

Comparative study between the diabetic non-calculogenic and diabetic calculogenic showed maximum crystal growth in the diabetic calculogenic rat urine added set (Fig.1).

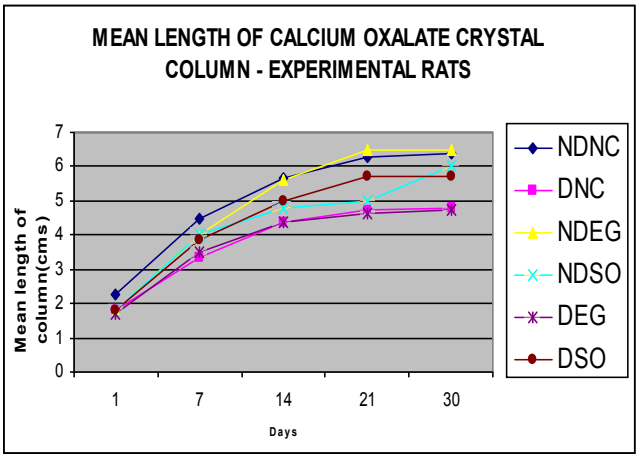

Fig.1. Graphic and Tabular Representation Showing Mean Length of calcium Oxalate Crystal Column – Experimental Rats

Days	ND NC	D NC	ND EG	ND SO	D EG	D SO
1	2.25	1.82	1.80	1.80	1.70	1.80
7	4.47	3.35	4.00	4.00	3.50	3.85
14	5.65	4.37	5.60	4.80	4.35	5.00
21	6.30	4.72	6.50	5.00	4.65	5.70
30	6.40	4.77	6.50	6.00	4.75	5.70

ND NC: Non-diabetic non-calculogenic
D NC: Diabetic non-calculogenic
ND EG: Non-diabetic ethylene glycol induced calculogenic
ND SO: Non-diabetic sodium oxalate induced calculogenic
D EG: Diabetic ethylene glycol induced calculogenic
D SO: Diabetic sodium oxalate induced calculogenic

Analysis of Variance showed significant difference in mean values among the groups. Size of the calcium oxalate crystal *in- vitro* showed significant difference in mean (p=0.001). Highest mean of 398.75± 1.25µ was seen in the diabetic sodium oxalate induced calculogenic rats compared to the other groups on most of the days assessed.

Table 1. Mean value, F ratio and Duncan's Multiple Range Test of the mean size of calcium oxalate crystals *in-vitro* weekly in different groups of experimental rats

Groups	Day 1 Mean ± SE (µ)	Day 7 Mean ± SE (µ)	Day 14 Mean ± SE (µ)	Day 21 Mean ± SE (µ)	Day 30 Mean ± SE (µ)
ND NC	191.00 ± 0.57 *e*	270.25 ± 3.48 *d*	313.13 ± 3.73 *d*	335.00 ± 2.04 *b*	359.75 ± 2.75 *b*
D NC	51.56 ± 2.94 *a*	159.38 ± 9.79 *a*	211.50 ± 5.77 *a*	261.88 ± 13.94 *a*	274.62 ± 14.12 *a*
ND EG	153.75 ± 1.35 *d*	234.50 ± 5.17 *c*	273.38 ± 2.21 *b*	295.88 ± 4.12 *a,b*	303.75 ± 2.39 *a*
ND SO	94.50 ± 2.21 *b*	281.88 ± 1.69 *d*	293.00 ± 1.15 *c*	311.25 ± 1.49 *b*	353.50 ± 3.17 *b*
D EG	125.38 ± 2.17 *c*	210.63 ± 1.57 *b*	303.50 ± 2.36 *c,d*	266.25 ± 18.93 *a*	277.50 ± 17.50 *a*
D SO	168.13 ± 3.25 *d,e*	283.63 ± 2.62 *d*	329.50 ± 5.89 *e*	373.75 ± 2.39 *c*	398.75 ± 1.25 *c*
F	52.54	51.59	92.35	4.40	17.54
p	<0.001	<0.001	<0.001	<0.001	<0.001

Duncan's Multiple Range Test with significance level at 0.05
a: Homogeneous Subset 1
b: Homogeneous Subset 2
c: Homogeneous Subset 3
d: Homogeneous Subset 4
e: Homogeneous Subset 5

When Duncan's Multiple Range Test was applied, at the end of one month, the mean size of the crystals was maximum in the diabetic sodium oxalate induced calculogenic rats compared to other groups. Maximum crystal growth seen in the non-diabetic calculogenic urine added set indicates the presence of promoters of crystallization in the urine of calculogenic rats. Comparative study between the diabetic non-calculogenic and diabetic calculogenic (ethylene glycol and sodium oxalate induced) rats showed maximum length of crystal column in the diabetic ethylene glycol induced calculogenic urine added set. This indicates the tendency of diabetes along with calculogenesis for promoting maximum crystal growth. One way analysis of the size of the calcium oxalate crystals in-vitro weekly on adding the urine samples of the non-diabetic and diabetic non-calculogenic, non-diabetic and diabetic calculogenic (ethylene glycol and sodium oxalate induced) group of rats showed significant difference ($p<0.001$) in the size of the crystals on all days. The maximum size of crystals seen in the diabetic sodium oxalate induced calculogenic rats on days 7, 14, 21 and 30 indicates the tendency of diabetic calculogenic rats for stone formation. Duncan's Multiple Range Test showed significant difference in the mean size of the crystals between the groups on almost all the days. Maximum size of the crystals was seen in the diabetic sodium oxalate induced calculogenic rats (Table 1). This indicates that diabetes along with calculogenesis can promote calcium oxalate crystallisation.

Conclusion

In-vitro study of the growth of calcium oxalate crystals on adding urine samples of experimental rats showed maximum crystal growth in the set adding urine of diabetic sodium oxalate induced calculogenic rats. The mean length of calcium oxalate crystal column in-vitro was maximum in the diabetic urine added set. This concludes that diabetic calculogenic urine contains promoters of crystallization and the tendency of diabetes along with calculogenesis for promoting maximum crystal growth.

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