Original Research Article

Study of Cystatin–C and its association with urinary citrates in diabetic Nephropathy

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Abstract

Diabetic nephropathy is occurs due to severe complication of diabetes mellitus and its has been identified as a risk factor for cardiovascular disease (CVD) related outcomes including mortality heart failure, myocardial infarction and stroke . Metabolic changes associated with diabetes lead to glomerular hypertrophy, glomerulosclerosis, and tubule-interstitial inflammation and fibrosis. There is more risk of diabetic renal disease onset and progression with current therapies for diabetes mellitus. Hence, widespread innovation is immediately needed to improve health outcomes for patients with diabetic renal disease and can reduce chances of diabetic nephropathy. Cystatin-C is a 13 kDa cysteine protease inhibitor that is produced by cells throughout the body. Cystatin-C is abundant in serum/plasma and is readily filtered at the glomerulus. Serum levels of cystatin-C have been proposed as a useful marker to estimate glomerular filtration rate. So the the present study is aimed to evaluate the possible association between cystatin–C and urinary citrate along with other biochemical parameters in diabetic nephropathy patients. A total 46 diabetic nephropathy patients and 46 healthy ages matched; individuals between the ages of 35 to 45 years were selected for this study. All the parameters are analyzed by conventional standardized methods and compared between the two groups. The student 't' test was applied for the statistical analysis and the results were expressed in mean ± SD and $p < 0.001$ were considered as highly significant. The mean levels of cystatin –C levels were increased ,urinary citrates are significantly reduced and micro protein, urinary protein creatinine ratio and micro-albumin are significantly elevated in the diabetic nephropathy patients when compared to control group.

Keywords: Diabetic nephropathy, Cystatin-C, urinary citrates, micro protein and microalbumin

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Introduction

Diabetic nephropathy (DN) is a type of progressive renal disease mainly affects ~40% of people with type-1 and type -2 diabetes [1]. DN is considered as main reason for issues like end stage renal disease (ESRD) and chronic kidney disease. The recent estimation or survey conducted by International Diabetic Federation and KDIGO on 2020 shows that, the prevalence of diabetes is about 10.9% [2]. It is well known that, chronic complications results in diabetes mellitus (DM). The organs damage initiated by DM is depends on the intensity and time period of hyperglycemia.

Cystatin C is a alkaline secreted protein , potent inhibitor of cysteine proteases with a molecular mass of 13.343 Da, which could strongly inhibit the activities of papain-like cysteine proteases and legumain . Cystatin C encoding gene was once believed to be the house-keeping gene found in all of the nucleated cells without tissue specificity [3,4]. The assessment of Cystatin C is mostly used as a biomarker of renal functions due its lower molecular weight and easier detection to measure glomerular filtration rate (GFR) than chemical compounds, radioisotopes or radiocontrast agents [5,6]. The end stage of renal failure is followed with a progressive excretion of Urine albumin, followed with blood pressure, decrease in glomerular filtration [7]. It is also found and reported that the relation between DN and diabetic cardiopathy means chance of cardiovascular diseases increases with increase of albuminuria and hence throughout the world DN is considered as the reason for final stage of renal failure [8]. The other associated factors for DN are more retinopathy, earlier cardiovascular diseases, high blood pressure, dyslipidaemia considered as factors for DN [9]. The control or prevention of DN is possible with the control of blood pressure and glucose level. In the study of DN, the National Kidney Foundation in association with American Diabetic Association recommends the screening of micro albuminuria in type-1 and 2 DM patients for longer period [10]. Factors like urinary albumin excretion rate, serum creatinine, renal biopsy, glomerular filtration rate, minimal proteinuria, time duration of diabetes mellitus, active urine sediment with red blood cells casts in urine will help much in diagnosis of DN. Along with the above, presence of sodium, potassium, urea, creatinine will help to decide the stage of DN [11]. So in the present study we aimed to find out the association between cystatin –C and urinary citrate along with other biochemical parameters in diabetic nephropathy patients.

Materials and methods

The duration of this study was from Feb- 2019 to April-2020, Forty six adult Male & female recurrent diabetic nephropathy patients, between the ages of 35 to 45 years were considered as study group who underwent treatment in Andra Medical College, Visakhapatnam, Andra Pradesh. Forty six healthy volunteers of matched age with no chronic renal failure or positive history of renal stones, or evidence of hypertension and diabetes, were included as controls group of the study. Patients with clinical history like tuberculosis, hemorrhage, urinary tract infection and renal impairment were avoided from the study. The detailed history of both groups has taken and appropriate physical examination was done. The participants of each study received verbal and written instructions about collection of 24 hour urine sample in a special container. During the experimental procedure according to the Declaration of Helsinki 1975, the Institutional Ethical Committee clearances were obtained.

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Analytical methods

All samples were tested in the same laboratory. For the purposes of this study, Serum Cystatin C (Latex enhanced Immunoturbidimetry /AGAPPE) were estimated using UV Spectrophotometer (Systronics). Serum creatinine (Mod. jaffe's Kinetic method), blood urea (Modified Berthelot Method) using Erba EM-200 fully automation and Serum electrolytes using ST-200 electrolyte analyzer and for all control and study subject, routine urine analysis was carried out. Urine was collected from study and control group in a container with 10ml of 10N Sulfuric acid as preservative over period of 24 hours and urinary citrate excretion was measured. Micro albumin carried out by sandwich immune detection method using one step MAU rapid quantitative test is based on fluorescence immunoassay technology. Micro protein was determined by pyrogallol red method (end point), urine protein and creatinine ratio using calculation formula. The urinary citrate excretion was assayed by the colorimetric method with pentabromoacetone (PBA). Citric acid is oxidized to PBA by bromine and the formed PBA is extracted with ether and reacts with borax buffer solution to form yellow color, and measured using spectrophotometer at 445nm [12].

Statistical analysis: The statistical analysis was carried out using software 25.0 of Statistical Package for Social Sciences. The statistical analysis was done by using student 't' test and the results were expressed in mean ± SD, where the p values ($p < 0.001$) were considered as highly significant.

Results and Discussion

Table 1: Mean ± S.D of parameters in Diabetic nephropathy patients and control groups

Parameters (n=46)	Control group **Mean ± SD**	Diabetic nephropathy (DN) patients **Mean ±SD**
FBS (mg/dl)	85.31 ± 14.2	193.9 ± 18.44*
PPBS (mg/dl)	104.3 ± 8.185	257.7 ± 59.38*
HbA_1c	5.9 ± 0.3	8.613 ± 0.8*
Urea (mg/dl)	32.20 ± 10.12	82.33 ± 37.35*
Creatinine (mg/dl)	1.035 ± 0.246	5.347 ± 2.48*
Sodium (mmol/L)	136.45 ± 3.792	115.95 ± 23.25*
Potassium (mmol/L)	3.82 ± 0.275	5.55 ± 0.716*
Chloride (mmol/L)	103.3± 6.546	104±4.731*^{NS}

*Significantly different from control at P <0.001, NS: Not significant

Table 2: Mean ± S.D of special Parameters in Diabetic nephropathy patients and control groups

Parameters (n=46)	Control group **Mean ± SD**	Diabetic nephropathy(DN) patients **Mean ±SD**
Cytatin –C	0.64 ±0.08	1.4 ±0.16*
Micro albumin (mg/L)	13.65±4.12	39.82±13.96*
Micro protein (mg/day)	78.6 ± 26.77	198.4 ±39.33*
Protein /Creatinine ratio (mg/gm)	0.22 ± 0.07	3.38 ± 0.518*
Urinary citrate (mg/24hr urine)	334.8 ± 13.45	291.9 ± 17.74*

*Significantly different from control at P <0.001

Table 3: **Correlation between Serum Cystatin -C & measured parameters in diabetic nephropathy patients**

Parameters	Correlation Coefficient-r	p –value
Micro albumin	0.349	0.01**
Micro protein	0.247	0.04*
Protein /Creatinine ratio	0.423	0.002**
Urinary citrate	-0.356	0.003**

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

The control has 46 healthy individuals and DN group has 46 patients. The mean age of the patients and individuals in Study and Control Group was between 35 to 45 years. In normal individuals (control group), fasting blood sugar (FBS), postprandial blood sugar (PPBS) mean level was 85.31 ± 14.2 mg/dl, 104.3 ± 8.185. In DN group patients, the FBS, PPBS was significantly raised being 193.9 ± 18.44 mg/dl & 257.7 ± 59.38 respectively. The mean HbA1c in control is 5.9 ± 0.3mg/dl, while the mean value of HbA1c in DN patients is 8.613 ± 0.8mg/dl. HbA1c slightly increases in DN patients are given table-1. The average glycemia for the past 6 to 8 weeks by the HbA1c test and it is well known that, glycation of hemoglobin occurs over the entire 120 days life span of RBCs but recent glycemia has the largest influence on the HbA1c value during these 120 days [13]. The HbA1c value more than 6.5 in study group patient show the poor glycemic control, in spite of insulin treatment. The glycation of proteins like HbA1c and increased free radical generation could promote the development of further complications.In normal individuals (control group) blood urea & creatinine mean levels were 32.20 ± 10.12 mg/dl and 1.035 ± 0.246 mg/dl respectively. Both urea and creatinine was significantly raised being 82.33 ± 37.35mg/dl and 5.347 ± 2.48 mg/dl respectively in diabetic nephropathy patients are given table I.

In table-1, the serum electrolytes values in both groups are shown. The mean difference was statistically significant as the mean serum Sodium level in the study group and controls were 115.95 ± 23.25 and 139.3 ± 3.446 respectively. This finding is consistent and describe that patients with renal failure have impaired renal mechanism for conserving Na^+. The mean serum potassium level in the study group was 5.55 ± 0.716 when compare to the controls was 3.82 ± 0.275. Potassium is freely filtered through glomerulus & reabsorbed by PCT. Hyperkalemia is a frequent complication of renal failure, which is depending on the nature of pathology or the sites involved, a range of serum K^+ values are noted in kidney disease [14]. The mean serum chloride level in the study group was (104±4.775) and the controls are 103.3± 6.546.The mean difference was statistically not significant (P-0.246).

The DN also represents cause of chronic kidney disease (CKD) which leads to end stage renal disease (ESRD). The DN is considered as one of the major complications of DM. It is noticed that, DN complications found up to 40% in type I and type II DM patients. It is reported that DM is a major cause of CKD which leads to chronic renal replacement therapy (RRT) due to ESRD in western countries [15].

The management of diabetes was revolutionized during 1960s by the development of assays for detection of micro-albuminuria (MA). Till date, the MA is considered as an major biomarker for both glomerular and tubular injury used clinical index of DN and also associated with diabetic cardiopathy [16]. In western world, the DN is also considered, in diabetic cases, if the albumin

excretion rate is more than 300mg over a period of 24 hours, and also a decline in renal function [17]. In this study, the mean micro albumin values in control group and the study group were 13.65±4.12 and 39.82±13.96 respectively. It is noticed that, the mean micro albumin value is significantly increased in study group. It was reported that, a reduction of Urinary albumin excretion in type 2 diabetic patients reflects the reduction of renal and cardiovascular risk. Also the MA is considered as an indicator of widespread micro-vascular disease and of underlying renal disease [18]. In DM, proteinuria has been considered as one of the earliest signs of renal function deterioration. It is known that, failure in the reabsorption of filtered proteins in the tubular cells and also alterations in glomerular permeability resulting in proteinuria formation. Generally on an average, healthy adults excrete 20-150 mg of protein in urine over a period of 24 hours. High risks of renal and cardiovascular diseases like nephrotic syndrome, chronic nephritis, lupus nephritis, hypertensive nephropathy, diabetic nephropathy are associated with the presence of urinary proteins [19]. Table -2 shows the mean Cytatin –C levels in study group (1.4 ±0.16) increased than compared to control group (0.64 ±0.08). Micro protein level in the study group was increased (198.4 ±39.33) when compared to the control group (78.6 ± 26.77) and the mean difference was statistically significant. The urine Protein Creatinine Index (PCI) is another method in which the creatinine excretion is generally constant in any subject over a period of 24 hours and it correlates with the 24 hour total excretion of the protein [20-26]. The mean difference was statistically significant as the mean PCI increased in the study group 3.38 ± 0.518 when compared with controls 0.22 ± 0.07.

The 24 hour urinary citrate mean values in control group were 334.8 ± 13.45 where as in study group it is 291.9 ± 17.74. The mean value of urinary citrate is significantly decreased in DN group. Urinary citrate values in both groups are as shown in table 2. By applying two samples to *t*- test on citrate values in all participants in both groups, 'p' value was < 0.001 which is statistically significant.

Table-3 shows a significant positive correlation between Cystatin-C and microalbumin, microprotein , protein creatinine ratio and negative correlation between urinary citrates. The urinary citrate excretion is depends upon the urinary volume, calcium, magnesium excretion and GI-alkali load [18]. Ghys et al. 2014 reported that Cystatin C is an marker of both ischemic and nephrotoxic renal injury and assessment of serum cystatin C as an alternative to traditional markers such as BUN and creatinine for the estimation of glomerular filtration rate and sensitive indicator of proximal tubular injury [27]. In the present study we observed the decreased excretion of citrates in DN patients compared to healthy volunteers and shows a significant negative correlation between cystatin c and urinary citrates. It is also possible by high intake of meat increases the urinary excretion of calcium, oxalate, and uric acid and decreases urinary pH and citrate excretion [23,28]. The risk of stone formation is possible when the intake of high-protein and low-carbohydrate diets for weight loss, as these are associated with decreased urinary citrate, pH levels and increased urine calcium and sodium levels [24]. There is an increasing risk of Calcium nephrolithiasis when hypocitraturia enhances urine calcium salt super saturation and also reduces calcium crystallization inhibition. It will play a role in solubility of uric acid and uric acid stone formation [25]. The dysfunction of the sodium citrate co-transport or disorder red intracellular citrate regulation etc are the secondary intrinsic renal defects caused by hypocitraturia in appropriate intestinal citrate or alkali absorption, or a normal physiologic response to animal protein- rich diets [26]. The major outcomes of CRF, as well as ARF to an extent, the specific diagnosis of a type of kidney disease, which includes progressive kidney failure and complications from reduced function. The early detection followed by treatment may delay or prevent the adverse outcomes

Conclusion

In the study of diabetic nephropathy, we analyzed different biochemical parameters in DN patients as well as in controls. It is concluded that, in DN patients, the variation in Cystatin-C micro-albumin level, urine proteins, urinary protein creatinine ratio, urinary citrates and the like are very important diagnostic tools in addition to prognosis. Regular monitoring of Cystatin C for diabetic patients will be useful for early diagnosis of renal failure, followed treatment will help in reducing the rate of morbidity and delayed fatality of chronic renal diseases and hence, DN patients may benefit. During the above study in DN subject, there is a noticeable difference in urinary citrate factors in these two control and study groups. This type of studies, will help in predict and hence increases the scope of early diagnosis, prognosis and in therapy.

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