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Original Article

Biochemistry Section

Study of Urinary Glycosaminoglycans among Essential Hypertensive Patients

IC DEVARAJ[1], DHIRAJ J TRIVEDI[2], VIDYA S PATIL[3], V INDUMATI[4], NB SANJEEVINI[5]

ABSTRACT

Introduction: Essential hypertension is a systemic disease which affects endothelial basement membrane. Changes in Glycosaminoglycans (GAG) distribution pattern on glomerular basement membrane has been noted in hypertension. Hence it seems likely that an increase excretion of GAG levels may be an indicator of reduction in renal function. The qualitative or quantitative determination of urinary GAGs may be of value and could constitute a non invasive marker to assess the renal damage in essential hypertension.

Aim: Differential analysis of urinary GAGs in essential hypertensive patients.

Materials and Methods: This analytical case-control observational study which was conducted from November 2014 to June 2016 in Department of Biochemistry, SDM College of Medical Sciences and Hospital, Dharwad, Karnataka, India. The study group included 50 male patients with age group of 30-60 years, clinically diagnosed with essential hypertension. Control group included 50 healthy male individuals with age group of 30-60 years, blood donors visiting to hospital blood bank. Random urine sample was collected. Urine creatinine was estimated by Jaffe's method, urine GAGs by Dimethyl methylene blue (DMMB) dye method and urine microalbumin by particle enhanced Turbidimetric inhibition immunoassay method. Correlation was tested with Spearman's correlation coefficient. Level of significance was set for p-value <0.05 with confidence interval of 95%.

Results: In present study, urinary GAGs levels in essential hypertensive study group was 14.57±10.16 mg/dL and in control group was 10.09±6.04 mg/dL. Urinary GAGs levels in essential hypertensive group was significantly high (p-value=0.890) when compared with normotensive control group. But no statistical significant correlation was found between urine GAGs and urine microalbumin in hypertensive study group.

Conclusion: Estimation of urine glycosaminoglycans in essential hypertensive patients is a simple, rapid and cost effective test which asseses the glomerular function. It can be used as one of the early marker for diagnosis of nephropathy before microalbuminuria sets in.

Keywords: Basement membrane, Creatinine, Glycocalyx, Kidney, Microalbuminuria

INTRODUCTION

Prevalence of essential hypertension is increasing over the years in India affecting mainly young population. Uncontrolled and persistent hypertension can affect the heart leading to ischaemic heart disease, affects blood vessels leading to peripheral vascular diseases, affects brain leading to transient ischaemic attacks and stroke, affects the eyes leading to retinopathy and affects kidneys leading to nephropathy. After diabetes mellitus, hypertension is one of the major disease that can cause impairment of renal function.

Hypertension is defined as a systolic blood pressure above 140 mmHg and/or a diastolic blood pressure above 90 mmHg [1]. Essential hypertension is a hypertension without a known cause, also called as idiopathic/primary hypertension. Among the hypertensive patients, 95% have essential hypertension. It is associated with impaired endothelial mediated vasodilatation which affects the functioning of resistance vessels [2].

Routinely performed tests to assess renal function in biochemistry laboratory are serum urea, serum creatinine and urinary microalbumin. Urea is an end-product of protein metabolism produced in liver, which forms a normal constituent of urine excreted through the kidneys. Blood urea does not raise in plasma until Glomerular Filtration Rate (GFR) reduces to 50-60% and it is also influenced by dietary intake of protein.

In healthy muscles creatinine is a spontaneous, non enzymatic end product of creatinine phosphate metabolism. Creatinine produced in muscles is released in circulation and transported to kidneys for excretion in urine. Amount of 24-hour urine creatinine excretion is correlated with muscle mass and it is constant for given individuals. Once creatinine is filtered through glomerulus it is neither secreted nor reabsorbed in tubular system. It forms normal constituent of urine. It is a measure of glomerular function of kidney. Estimation of creatinine is more sensitive index for renal function than the blood urea but, it also varies according to muscle mass. Additionally, certain non renal conditions affects serum and urine creatinine levels. Albumin is the most abundant plasma protein in the body. In normal subjects though small amount of albumin is filtered through the glomerulus, almost all gets reabsorbed by renal tubules, only very negligible amount less than 30 mg/day is excreted in urine. When urine albumin excretion is 30-300 mg/day it is called as microalbuminuria. It is an established marker of nephropathy but microalbuminuria occurs only when there is significant renal damage [3].

In detecting early stages of renal damage above renal markers are insensitive. Hence, there is a need to search for an early markers of renal damage. Glycosaminoglycans are polyanionic, unbranched, heteropolysaccharides, rich in sulphate groups. They are present over glomerular basement membrane of the kidney. Sulfate moieties of Glycosaminoglycans (GAG) play an important role in glomerular charge permeability for proteins present in plasma. Proteinuria is due to an alteration of the glomerular charge-selective and size-selective barrier of podocytes [4].

In essential hypertension there is increased arterial pressure which have direct effect on Glomerular Basement Membrane (GBM) causing excretion of GAGs. Urinary loss of GAGs has been associated with increased albumin permeability and their excretion in urine [5]. Normally, urinary GAGs excretion is in the range of 50.11 mg/24 hr [6] or 1.98 to 5.94 mg/dL [7]. Long standing essential hypertension causes arteries around kidneys to narrow, weaken or harden. This leads to decrease in glomerular filtration rate later causing renal damage.

Routine renal markers can indicate damage only after some percentage has occurred. It is of great value if this renal damage can be detected at an early phase, so aim of this study to estimate urine GAGs may be a hope. Hence, the quantitative determination of urine GAGs by dimethyl methylene blue method and its correlation with urine microalbumin may provide us a simple, cost effective, non invasive marker to assess the renal damage at early phase in essential hypertensive individuals. Study was proceeded with following objectives:

1. To compare urinary excretion of GAGs in essential hypertension with normal healthy individuals.
2. To correlate the urinary excretion of GAGs with microalbuminuria among essential hypertensive patients.

MATERIALS AND METHODS

This was a analytical case-control observational study, conducted for two years from November 2014 to June 2016 in Department of Biochemistry, SDM College of Medical Sciences and Hospital Dharwad, Karnataka, India. This study was approved by Institutional Ethical Committee. (Ref No: SDMIEC: 0424: 2014 Dated: 7/11/2014). It has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Total one hundred subjects were included in the study.

Sample size calculation: Sample size calculation was done from a software www. Openepi.com. Among them hypertensive group comprised of 50 male individuals, diagnosed as essential hypertensive in the age group of 30-60 years, attending medicine outpatient department. Again these hypertensive group were divided in to two groups based on duration of Hypertensive (HTN)- Group B (<5 years of HTN), Group C (>5 years of HTN). Normotensive control group (Group A) comprised of age matched healthy 50 male blood donors visiting medical college blood bank. Individuals in study group were selected randomly based on their clinical history and recording of blood pressure.

Informed consent was obtained from each individuals who were willing to participate in the study.

Inclusion criteria:

- Hypertensive (HTN) group: Clinically diagnosed (systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg) [1].
- Essential hypertensive male patients within the age group of 30-60 years.
- Normotensive (NT) Control group: Male blood donors within the age group of 30-60 years and having normal blood pressure.

Exclusion criteria:

- Males below 30 years and above 60 years of age.
- Females.
- Patients with Secondary hypertension.
- Diabetes mellitus.
- Fever, renal stones, thyroid abnormality, glomerulonephritis.

Based on questionnaire and blood pressure recording, essential hypertensive male patients within the age group of 30-60 years were selected and patients with secondary HTN were excluded. Study population were divided into 3 groups:

Group A: Comprised of healthy normotensive individuals (50 male individuals).

Group B: Comprised of hypertensive individuals with less than 5 years of duration {38 (76%) male individuals}.

Group C: Comprised of hypertensive individuals with more than 5 years of duration {age matched 12 (21%) male individuals}.

Group B+C: Total 50 hypertensive individuals with systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg, attending Medicine Outpatient Department.

Method for Collection of Urine Sample

Every individual was instructed to collect 100 mL of random, midstream urine sample in a sterile plastic container without any preservatives between 9 AM to 12 PM. Container was stored in refrigerator till analysis on same day. Urine sample was analysed without any centrifugation. The biochemical estimations of urine creatinine, microalbumin and glycosaminoglycans were performed in Department of Biochemistry laboratory on the same day.

- Estimation of urine creatinine by Jaffe's method [8]: Concentration of urine creatinine was read from standard graph and values were expressed in mg/dL.
- Estimation of urine GAGs by Dimethyl Methylene Blue (DMMB) dye method [9]. Based on a principle that DMMB, a thiazine chromotrope which on binding to sulphated GAGs at pH 3 produces purple colour leading to change in absorption spectrum due to induction of metachromasia, enabling rapid detection of GAGs in solution. Concentration of urine GAGs was read from standard graph and the values were expressed in mg/dL.
- Estimation of urine microalbumin by particle enhanced Turbidimetric inhibition immunoassay method [10]. Concentration of microalbumin in urine was expressed in mg/L.

STATISTICAL ANALYSIS

All results obtained was statistically analysed by Statistical Package for the Social Sciences (SPSS) version 20.0 software. Descriptive statistics were analysed by Chi-square test. Data of urine GAGs, urine microalbumin and urine creatinine was expressed in terms of mean±SD. Mann Whitney U test was used to compare non parametric tests. Correlation was tested with Spearman's correlation coefficient. Level of significance was set for p-value <0.05 with confidence interval of 95%.

RESULTS

Mean age of group A was 44 years, group B was 50 years, group C was 56 years. Mean value of urine GAGs in group A was 10.09±6.04 mg/dL in group B was 13.62±9.4 mg/dL and group C as 17.58±12.22 mg/dL. Significantly higher level (p-value=0.0269) of GAGs was found in HTN group (B+C) as compared to NT (group A). Similarly, mean value of urine microalbumin in group A was 12.47±7.36 mg/L, in group B was12.48±19.05 mg/L and in group C was 25.63±17.48 mg/L. There was no significant difference observed between HTN and NT groups. Also, mean value of urine creatinine in group A was 93.01±43.76 mg/dL, in group B was 109.74±64.86 mg/dL and in group C was 141.72±75.29 mg/dL. There was no significant difference observed between HTN and NT groups [Table/Fig-1].

Parameters expressed in Mean±SD	Group A (n=50)	Group B (n=38)	Group C (n=12)	B+C (n=50)	Mann Whitney U test (B+C)	p-value (B+C)
U.GAGs (mg/dL)	10.09±6.04	13.62±9.4	17.58±12.22	14.57±10.16	929.0	0.0269*
U.microalbumin (mg/L)	12.47±7.36	12.48±19.05	25.63±17.48	15.64±19.3	1206.00	0.7616
U.creatinine (mg/dL)	93.01±43.76	109.74±64.86	141.72±75.29	117.41±68	1013.5	0.1030

[Table/Fig-1]: Comparison of urine GAGs, urine microalbumin and urine creatinine among group A and group B+C by Mann Whitney U test.
*p<0.05 considered significant; U: Urine

When urine GAGs, urine microalbumin and urine Creatinine in Group B was separately compared with Group A it shows Group B has no statistical significant difference [Table/Fig-2].

Parameters expressed in Mean±SD	Group A (n=50)	Group B (n=38)	U-value	p-value
U.GAGs (mg/dL)	10.09±6.04	13.62±9.4	750	0.092
U.microalbumin (mg/L)	12.47±7.36	12.48±19.05	766.5	0.123
U.creatinine (mg/dL)	93.01±43.76	109.74±64.86	840	0.357

[Table/Fig-2]: Comparison of urine GAGs, urine microalbumin and urine creatinine between group A and group B by Mann Whitney U test.
p<0.05 considered significant; U: Urine

When GAGs in Group C were separately compared with Group A it shows Group C has (p-value=0.03) significant elevated GAGs level, indicating prolonged duration of HTN has more impact on the excretion of GAGs. Similarly where duration of disease is more, microalbumin shows almost double the value in Group C compared to Group A having high statistical significance (p-value=0.013). Same result is obtained for creatinine which shows significant difference (p-value=0.025) of Group C compared with Group A [Table/Fig-3].

Parameters expressed in Mean±SD	Group A (n=50)	Group C (n=12)	U-value	p-value
U.GAGs (mg/dL)	10.09±6.04	17.58±12.22	179	0.031*
U.microalbumin (mg/L)	12.47±7.36	25.63±17.48	160.5	0.013*
U.creatinine (mg/dL)	93.01±43.76	141.72±75.29	173.5	0.025*

[Table/Fig-3]: Comparison of urine GAGs, urine microalbumin and urine creatinine between group A and group C by Mann Whitney U test.
*p<0.05 considered significant; U: Urine

In NT (Group A) group when traditional marker of nephropathy, urine microalbumin was correlated with creatinine and GAGs it shows no significant correlation, but when the proposed marker, GAGs correlated with creatinine and microalbumin, it shows statistical significant correlation with creatinine but not with microalbumin.

Similarly in HTN (Group B+C) group, when microalbumin was correlated with urine creatinine and GAGs it does not shows any statistical significance but, significant correlation was observed between creatinine and GAGs with rho-value 0.321 and p-value=0.023 [Table/Fig-4].

Groups	Variables	Correlation between GAGs (mg/dL) with		
		Spearman R	t-value	p-value
NT group (Group A) (n=50)	Creatinine (mg/dL)	0.5550	4.6229	0.0001*
	Microalbumin (mg/L)	-0.1958	-1.3830	0.1731
HTN group (Group B+C) n=50	Creatinine (mg/dL)	0.3210	2.3481	0.0230*
	Microalbumin (mg/L)	0.0199	0.1379	0.8909

[Table/Fig-4]: Spearman's rank correlation of urine GAGs (mg/dL) with urine creatinine (mg/dL) and urine microalbumin (mg/L) in NT (group A) and HTN (group B+C) group.
*p<0.05 considered significant; HTN: Hypertensive; NT: Normotensive

DISCUSSION

Glycosaminoglycans play an important role in permeability of glomerular basement membrane. Increased loss of GAGs from GBM leads to reduction of charge selectivity causing urinary loss of albumin. Nephropathy is a common complication in many disorders out of which hypertension and diabetes are major contributors. By the estimation of GAGs in these conditions early detection of nephropathy may be possible before microalbuminuria sets in.

In the present study, urine GAGs level were 14.57±10.16 mg/dL and 10.09±6.04 mg/dL in essential hypertensive group and in normotensive group respectively. Urine GAGs levels were significantly higher in essential hypertensive group compared to normotensive group. When duration of HTN was compared it shows that persons having <5 years of history has 13.62±9.4 mg/dL as compared to 17.58±12.22 mg/dL in persons having >5 years of history. This result shows that as duration of HTN increases it has more impact on GBM and results in increased excretion of GAGs showing significance of p-value=0.031.

Urine microalbumin level was 15.64±19.36 mg/L and 12.47±7.36 mg/L in essential hypertensive group and in normotensive group respectively. There was no statistical significant difference of urine microalbumin level between essential hypertensive group and normotensive group. When duration of HTN was compared it shows that persons having <5 years of history has 12.48±19.05 mg/L as compared to 25.63±17.48 mg/L in persons having >5 years of history. This result shows that in initial five years microalbumin levels are not affected but, as duration of HTN prolongs it has shown significant increase (p-value=0.013) in value.

Urine creatinine level was 117±68.11 mg/dL and 93.01±43.76 mg/dL in essential hypertensive group and in normotensive group respectively. When duration of HTN was compared it shows that persons having <5 years of history has 109.74±64.86 mg/dL as compared to 141.72±75.29 mg/dL in persons having >5years of history. This result shows that urinary creatinine levels gradually increases as the disease prolongs. It becomes more significant (p-value=0.025) when Group C is compared with Group A.

There was no significant correlation found between urine GAGs and urine microalbumin but, urine GAGs and creatinine have shown significant correlationin group A (p-value=0.0001) and in group B+C (p-value=0.023).

As observed in the present study GAGs and creatinine are correlated well but microalbumin did not. This may be due to the initial phase of glomerular damage made small pore which are enough to pass through small molecular weight creatinine but restricting larger albumin molecules. As disease prolongs there is loss of more GAGs from GBM allowing even larger molecules like albumin at a later stage.

Yavuz D et al., observed increased GAGs excretion in newly detected hypertensive patients compared to control group, but no significant correlation between GAGs and microalbumin [5]. The finding was similar to the present results. As corroborative to present hypothesis Yasemin B et al., who hypothesised that, hypertension directly affects the GBM causing loss of glomerular anionic content, which may be associated with increased urinary GAG excretion [11]. As HTN advances it further leads to microalbuminuria.

Long standing DM is known to cause diabetic nephropathy. This may be due to damage caused on GBM by constant elevated blood glucose. Damage to GBM is reflected in the form of increased urinary GAGs as reported by Takamatsu UIK, Giovanni G and Juretic D et al., Poplawska-Kita A et al., and Antonio VG et al., all of them studied urinary GAGs excretion in diabetic patients and found higher excretion rate in diabetic study group compared to control, existing data coincides with the present findings [12-15]. Similarly in the present result there was no significant correlation between GAGs and microalbumin in early phase of diabetes but it was correlated as disease advances.

Proteinuria is an important sign in nephrotic syndrome. Cengiz N et al., studied GAGs excretion in group of children with nephrotic syndrome [16]. They found significant increase in GAGs levels in children which is in agreement with the present finding. But in contrast their study GAGs excretion significantly correlated with

severity of proteinuria which explains important role played by GAGs in nephrotic syndrome. This may be due to the study population age which is known to have elevated GAGs.

Contradictory results were also obtained in other studies on nephropathy. kaznowska-Bystryk I et al., studied in diabetic patients which showed lower levels of urinary GAGs in study group compared to control group [17]. They hypothesised that diabetes and hypertension induces glomerular endothelial inflammatory process. There is generation of nitric acid, which is metabolic product of nitric oxide causes reduced synthesis, enhanced turnover or enhanced degradation of GAGs leading to decreased levels of urinary GAGs. Though this explains the low levels of GAGs but, there is a lacuna about exact mechanism of loss of urinary GAGs. Many systemic and autoimmune diseases are known to cause membrane alteration by loss of glycocalyx. Estimation of GAGs excretion in urine may provide an index of the impact.

Cadaval RA et al., performed an experimental study in streptozocin induced rat, and developed HTN, DM and microalbuminuria in rat [18]. They reported urine creatinine was significantly low, urine microalbumin was significantly high and urine GAGs were low in these experimental rats as compared to controls.Though the results are contradictory to the present results because Streptozocin is known to cause diabetes and HTN which leads to mesangial deposition of GAGs as the earliest event before microalbuminuria set which could have reduced the excretion of GAGs in urine.

Extent of renal involvement in SLE significantly affects the outcome of disease. Parildar Z et al., studied urinary GAGs in SLE and found significant increased excretion of GAGs in the study group compared to controls [19]. Similar to present results they also found no correlation between GAGs and urine microalbumin.

De Muro P et al., studied urinary GAGs in chronic glomerulonephritis and found significant increased excretion of GAGs in study group compared to controls [20]. Suggesting that, in glomerulonephritis there is an antibody mediated glomerular injury causing increased GAGs excretion which later leads to alteration in glomerular permeability. In contrary to the present study, Tencer J et al., studied urinary GAGs levels in amyloidosis, they showed decreased excretion of urinary GAGs compared to controls which may be due to extracellular deposition of insoluble fibrillar proteins over glomerulus causing loss of functioning nephrons [21]. Tulay A et al., showed decreased excretion of GAGs in urolithiasis, they hypothesised that GAGs is a potent inhibitor of calium oxalate crystal formation and its aggregation [22]. Individuals with deficiency of GAGs are more prone for urolithiasis. According to Francyne kubaski et al methods like HPLC is sensitive but complex and time consuming. ELISA is rapid but expensive. So estimation of GAGs by dimethyl methylene blue method is cost effective [23].

Limitation(s)

Since sample size was less authors cannot generalise result on whole population. Also in this study, spot urine sample was taken to estimate GAGs, microalbumin and creatinine level, better results can be obtained if study was conducted on 24 hours urine sample.

CONCLUSION(S)

In present study the authors found significant higher levels of urine GAGs in essential hypertensive individuals. Urine microalbumin level was significantly increased as duration of hypertension increases. In early stages, urine GAGs was not correlated with urine microalbumin but with prolonged duration of HTN both were positively correlated. So further follow-up study of more than 5-10 years of hypertensive duration is required to find out correlation between urine GAGs and urine microalbumin and impact of HTN on GBM. Authors conclude that estimation of urine glycosaminoglycans in essential hypertensive patients is a simple, rapid and cost effective test which assess the glomerular function. Authors propose that it can be used as one of the early marker for diagnosis of nephropathy before microalbuminuria sets in. Glomerular involvement can occur not only in hypertension but various disorders like diabetes, nephrotic syndrome, glomerulonephritis and SLE so further study can be done to evaluate early glomerular damage in these disorders.

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PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Anaesthesiology, Vijayanagara Institute of Medical Sciences, Ballari, Karnataka, India.
2. Professor, Department of Biochemistry, SDM College of Medical Sciences and Hospital, Dharwad, Karnataka, India.
3. Professor and Head, Department of Biochemistry, SDM College of Medical Sciences and Hospital, Dharwad, Karnataka, India.
4. Professor, Department of Biochemistry, Vijayanagara Institute of Medical Sciences, Ballari, Karnataka, India.
5. Assistant Professor, Department of Biochemistry, Vijayanagara Institute of Medical Sciences, Ballari, Karnataka, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. NB Sanjeevini,
Assistant Professor, Department of Biochemistry, Vijayanagara Institute of Medical Sciences, Ballari-583104, Karnataka, India.
E-mail: sanjeevininb@gmail.com

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