

Progression of early phase diabetic nephropathy in streptozotocin-induced diabetic rats: Evaluation of various kidney-related parameters

G Kiran, CD Nandini, HP Ramesh & PV Salimath*

Department of Biochemistry and Nutrition, CSIR-Central Food Technological Research Institute (CFTRI), Mysore 570 020, India

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Diabetic nephropathy (DN) is one of the serious secondary complications of diabetes, which results in end-stage renal failure. Reports on the progressive nature of early phase DN especially with respect to kidney parameters such as kidney weight, type IV collagen excretion, total kidney and urinary glycosaminoglycans (GAGs) are few. This work was undertaken to determine systematically the progression of early phase DN in relation to various kidney-related parameters for a period of four months. Experimentally-induced diabetic rats were grouped based on fasting blood glucose levels. Various basic and kidney-related parameters such as kidney weight, microalbuminuria, urinary excretion of GAGs and type IV collagen, total kidney GAGs, histopathology, glomerular area and glomerular volume were examined in control and diabetic rats. There was a progressive increase in fasting blood sugar, urine sugar, kidney weight, microalbuminuria, urine glycosaminoglycans, urine type IV collagen, glomerular area and glomerular volume but there was a progressive decrease in kidney glycosaminoglycans. Glomerular sclerotic condition was aggravated with the increase in duration of diabetes from 1 to 4 months. Onset of DN in rats begins subtly after one month of diabetes but gets vitiated and more pronounced at the end of four months.

Keywords: Diabetes, Diabetic nephropathy, Glycosaminoglycans, Microalbuminuria, Type IV collagen

Diabetes, one of the metabolic disorders, is characterized by sustained hyperglycemia as a result of lack of insulin (Type 1). Diabetic nephropathy (DN) is one of the serious secondary consequences of diabetes resulting in end-stage renal disease¹. The multitude of problems associated with diabetes tends to magnify with its progression as a result of which monitoring becomes essential. Studies have shown that persistent high glucose levels in blood leads to increased oxidative stress², which is one of the causative factors for DN. Thus high blood glucose level is the main factor for initiation and progression of DN³.

DN results in the alteration of various extracellular matrix (ECM) components of kidney such as heparan sulfate (HS)⁴, laminin⁵ and type IV collagen⁶. This manifests itself as increased or decreased levels in serum and/or urine, which can serve as important markers for evaluating DN⁷. But it is not known as to at which stage these alterations start setting in. Also most of the time question arises as to what should be

the duration of diabetes in experimental models such as rat to observe nephropathic changes. This information is therefore essential to pinpoint the changes in relation to duration of diabetes.

The focus of this paper has been to systematically determine whether early phase DN over duration of 4 months leads to progressive changes in (a) basic diabetic parameters and (b) parameters associated with kidney such as kidney weight and kidney glycosaminoglycans, which can be correlated to diabetic condition and other markers such as albumin levels, type IV collagen excretion and GAG excretion.

Materials and Methods

Chemicals—Streptozotocin (STZ), heparin, dinitrosalicylic acid, 1,9-dimethylmethylene blue (DMMB), rat serum albumin (RSA), papain from *Carica papaya* and standard type IV collagen were obtained from Sigma (USA). Rabbit polyclonal antibody to type IV collagen was from Abcam (Cambridge, UK). Glucose estimation kit (enzymatic GOD-POD method) and creatinine test kit (modified Jaffe's reaction method) were from Span Diagnostic Ltd., Surat, Gujarat, India. Alkaline phosphatase-tagged goat anti-rabbit IgG secondary antibody, para

*Correspondent author

Telephone: +0091-821-2514876

Fax: +0091-821-2517233

E-mail: paramahans1954@gmail.com

nitrophenol phosphate (pNPP) and bovine serum albumin (BSA) were from Genei, Bangalore, India. All other chemicals and reagents used were of analytical grade.

Animals and diet—Male albino Wistar rats [OUTB-Wistar-IND cftri (2c)] weighing around 110-130 g were taken from the Institute's animal house facility. The study had the clearance from Institute Animal Ethical Committee. The animals were fed with AIN-76-based diet⁸.

Induction of diabetes—Diabetes was induced by a single intraperitoneal injection of STZ at 50 mg/kg body weight in freshly prepared citrate buffer (0.1 M, pH 4.5)⁹ and the control rats received citrate buffer only. Soon after STZ injection, 5% glucose water was given for two days, to prevent drug-induced hypoglycemic shock. Animals were caged individually having facilities to keep diet cup and water bottle. Food and water were provided *ad libitum*.

Grouping of rats—One week after the induction of diabetes, fasting blood glucose in blood drawn from retro-orbital plexus was determined. Control and diabetic rats were sub-grouped to enable monthly studies to be carried out by sacrificing control (n = 3) and diabetic rats (n = 4 or 5) every month for 4 months.

Urine and blood collection and harvesting of kidney—Urine was collected every month for a period of 24 h under a layer of toluene after keeping the rats in metabolic cages. Blood was collected in tubes containing sodium heparin salt (20 IU/mL) either from retro-orbital plexus during the experimental period or from heart puncture at the time of sacrificing the rats. Kidney was harvested, rinsed in cold saline and stored at -20°C until further analysis.

Isolation of GAGs—This was done according to Scott¹⁰. In brief, individual kidneys from both control and diabetic group were cut into small pieces and defatted in acetone for a week at 4°C and were powdered. They were subjected to papain digestion and centrifuged at 3000 rpm for 15 min at 4°C. To the supernatant one-third volume of 40% trichloroacetic acid was added to precipitate the proteins. It was centrifuged again and the supernatant which contains GAGs was precipitated out by adding 4 volumes of ethanol containing 1.2% potassium acetate. The precipitate was collected, dried using nitrogen gas and

reconstituted in minimum volume of water. It was quantitated by DMMB assay¹¹.

Estimation of type IV collagen—This was done by ELISA using 96-welled plates. Briefly, an aliquot of urine sample or standard type IV collagen was added to the wells and left overnight at 4°C. Primary antibody (Anti-type IV collagen, polyclonal) was added after blocking with 2% BSA for 4 h at room temperature. This was followed by incubation with ALP-conjugated secondary antibody (anti-IgG). The color developed by using pNPP as substrate was read at 405 nm in an ELISA reader. The amount of collagen excreted per day was calculated using the calibration curve generated.

Evaluation of albumin excretion by SDS-PAGE—Urine fraction equivalent to one minute was concentrated by speedvac. They were subjected to 10% SDS-PAGE for 6½ h at 50V and the bands were visualized by silver staining¹². Rat serum albumin (6 µg) was used as a standard.

Histology—Kidneys of control and diabetic rats were dissected out and fixed in 10% formaldehyde. The tissues were processed with graded ethanol, acetone and benzene and subjected to paraffin infiltration. Using rotary microtome, tissue sections of 5 µm thickness were obtained on slides and were stained with PAS dye. Photographs were taken at random in different fields under 400× magnification. Glomerular cross sectional area and glomerular volume was measured in atleast 10 glomeruli from each section using Image J software (NIH)¹³. Histopathological evaluation was done by blinded study and graded depending on the extent of damage.

Analytical methods—Fasting blood glucose in plasma was estimated by glucose oxidase method¹⁴, urine sugar by dinitrosalicylic acid method¹⁵, plasma and urine creatinine levels were measured by modified Jaffe's method¹⁶ and glomerular filtration rate (GFR) was measured using the formula as reported earlier¹⁷.

Statistical analysis—Statistical significance of the data between control and diabetic groups was evaluated by using unpaired, two-tailed Student's 't' test. Comparison was made between control and diabetic groups at I month duration 'a' and between I month and II, III and IV month diabetic rats 'b'. $P < 0.05$ was considered to be statistically significant. Correlation analysis was done by linear regression analysis using Microsoft excel.

Results

Effect of diabetes on basic parameters—All rats developed diabetes one week after injection of STZ, after which they were grouped based on fasting blood glucose levels. Rats were assessed for basic parameters every month for gain in body weight, diet intake, water intake, fasting blood sugar (FBS) and urine sugar for a period of four months (Table 1). Diabetic rats showed hyperphagia, polydipsia and polyuria with significant increases in fasting blood glucose and urine sugar levels compared to non-diabetic animals. The amount of diet consumed was more in diabetic animals but this did not result in weight gain when compared with controls.

Diabetes is known to affect kidney function. In order to determine the kidney function, glomerular filtration rate (GFR) in terms of creatinine clearance and microalbuminuria was estimated. GFR was significantly higher by 10 folds in diabetic animals compared to control ones in the first month itself. Subsequent months did not show any significant variation in GFR levels in diabetic animals (Table 1). Amount of albumin excreted in urine was higher in diabetic rats during 1 month of diabetes. At the end of 4 months, there was further increase in albumin excretion indicating worsening of diabetic condition. Electrophoretic examination of albumin on a 1 minute fraction of the urine sample from diabetic rats showed a progressive increase in the intensity of the band corresponding to the rat serum albumin (RSA) (Fig. 1a and b).

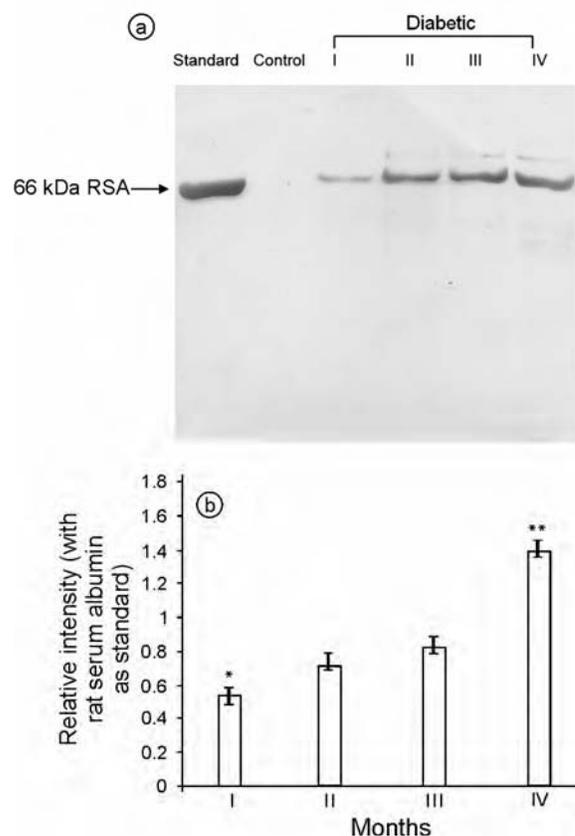


Fig. 1—Effect of duration of diabetes on albumin excretion. One minute urine fraction collected from control and diabetic rats of different duration of diabetes (I-IV) was subjected to SDS-PAGE and visualised by silver nitrate (a). Rat serum albumin (RSA), 66kDa was taken as standard. This was repeated in atleast 3 animals from each group. The relative intensity of bands (b) for the same was done using EASYWIN-32 gel doc software.

Table 1—Effect of duration of diabetes on basic parameters during I, II, III and IV month
[Values are mean \pm SD of 3 control and 4 or 5 diabetic observations from each group]

Basic parameters	Months							
	I		II		III		IV	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Body Weight (g)	238.6 \pm 12.4	134.6 \pm 17.2 ^a	311.6 \pm 9.2	175.6 \pm 26.5	348.3 \pm 17.5	185.7 \pm 47.3	372.0 \pm 31.3	151.2 \pm 33.2
Diet Intake (g/day)	12.8 \pm 1.7	22.1 \pm 3.9 ^a	14.9 \pm 2.3	16.2 \pm 2.0	11.63 \pm 0.40	18.9 \pm 3.2	16.0 \pm 1.5	25.0 \pm 2.6
Water Intake (ml/day)	25.0 \pm 8.6	105.0 \pm 5.0 ^a	15.0 \pm 0.0	64.0 \pm 15.4	15.0 \pm 5.0	102.5 \pm 29.5	26.6 \pm 5.7	105.0 \pm 29.7
FBS (mg/dL)	102.1 \pm 23.0	306.4 \pm 99.9 ^a	110.1 \pm 8.0	435.9 \pm 96.5	106.4 \pm 12.5	513.3 \pm 90.6 ^b	91.2 \pm 7.2	414.8 \pm 49.1
Urine sugar (g/day)	0.0013 \pm 0.0009	4.425 \pm 2.22 ^a	0.008 \pm 0.004	6.079 \pm 1.58	0.007 \pm 0.003	6.853 \pm 2.7	0.001 \pm 0.0004	7.058 \pm 2.02
GFR (mL/min)	0.2 \pm 0.1	5.3 \pm 1.5 ^a	0.3 \pm 0.1	5.5 \pm 1.2	0.8 \pm 0.1	4.1 \pm 1.0	0.3 \pm 0.2	5.5 \pm 0.3

P values: ^a< 0.05 & ^b< 0.05 represent comparison of I month control rats with diabetic rats and comparison between I month diabetic rats with II, III, IV month diabetic rats, respectively.

Effect on kidney weight and kidney GAGs—Kidney weight, measured in terms of weight of kidney/g body weight, increased when compared to control animals indicating the onset of renal enlargement which is a characteristic feature of diabetic rats. The increase was significantly higher in the first month when compared to control, remained stable at second and third months and showed significant increase at the end of fourth month (Fig. 2a). Kidney GAGs play a vital role in maintaining integrity of glomerular basement membrane. The amount of total GAGs in kidney showed a decrease in diabetic rats compared to controls. The decrease was significant in the first month between the control and diabetic groups. The decrease was not apparent at the end of second and third month, but was significantly decreased at the end of four months when compared to that of first month (Fig. 2b).

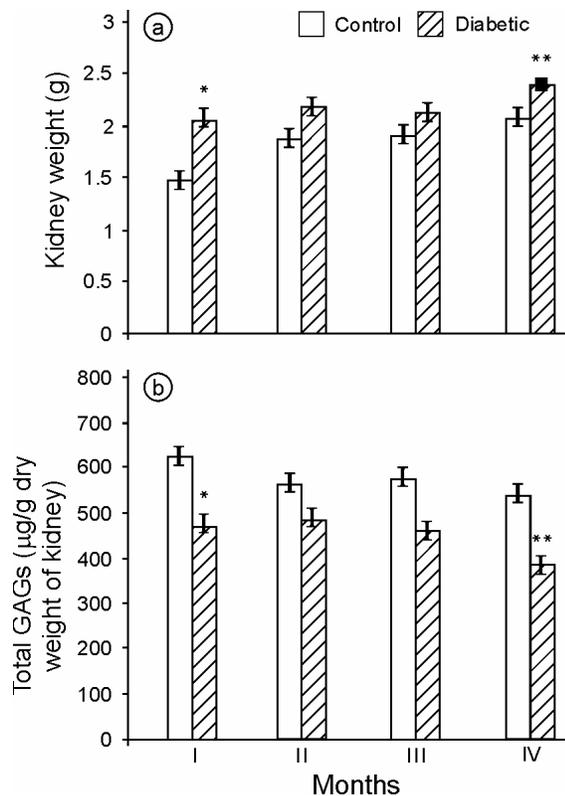


Fig. 2—Changes in kidney weight (a) and total kidney GAGs (b) during early phase DN. Values are given as mean \pm SD of control (n=3) and diabetic (n=4 or 5) rats. ***represent statistical comparison of I month control rats with I month diabetic rats and comparison between I month diabetic rats with II, III, IV month diabetic rats, respectively. A P value of ≤ 0.05 was deemed statistically significant. Closed and open bars represent control and diabetic rats, respectively.

Effect on excretion of GAGs and type IV collagen—Amount of urinary GAGs excreted increased in diabetic animals when compared to control rats. There was a progressive increase in excretion of GAGs in diabetic animals, which was significant at the end of four months (Fig. 3a). The increase observed was 8 fold higher compared to control rats which only worsened with increase in duration of diabetes.

Type IV collagen is one of the important ECM components present in kidney. The amount of type IV collagen in urine showed a progressive increase albeit on a subtler scale. Here again, diabetic rats showed higher amounts of type IV collagen levels in urine compared to control animals. Excretion reached statistically significant proportions at the end of four months when compared to first month diabetic animals (Fig. 3b).

Histopathological evaluation—Different grades of various pathological lesions were observed in the renal tissue of diabetic rats (Fig. 4). At the end of first month, two animals exhibited moderate distension of glomeruli while it was mild in three animals (Fig. 4b).

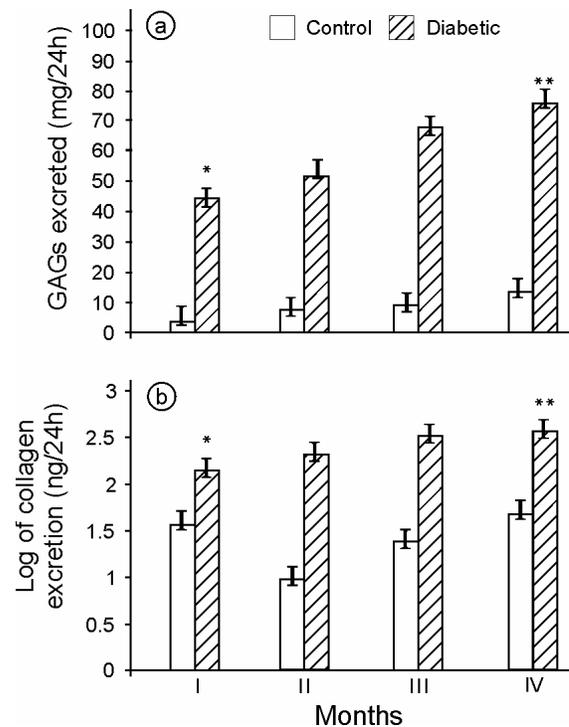


Fig. 3—Effect of diabetes on GAG and type IV collagen excretion. Excretion of GAGs (a) and type IV collagen (b). Values are given as mean \pm SD of control (n=3) and diabetic (n=4 or 5) rats. ***represent statistical comparison of I month control rats with I month diabetic rats and comparison between I month diabetic rats with II, III, IV month diabetic rats, respectively. A P value of ≤ 0.05 was deemed statistically significant.



Fig. 4—Histopathological evaluation of kidney sections. Formalin fixed kidney sections of 5 μ m thickness from control (n = 3) and diabetic (n = 4 or 5) were stained by PAS and images were taken at the magnification of 400 \times . Blinded study was done to determine glomerulosclerosis. Various panels represent control kidney (a), diabetic kidney–I month (b) and diabetic kidney–IV month (c).

Glomerular atrophy and mild loss of its cellularity were also seen in three animals. Glomerular symmetry was not normal in the experimental animals. One animal exhibited moderate fibrosis. Though, in the second and third months, histopathological status of glomeruli remained almost same, in the fourth month, glomerular distension was found to be severe in one animal, while two animals exhibited it moderately (Fig. 4c). Mild to moderate fibrosis was also seen in experimental animals.

Morphometric analysis of PAS-stained kidney sections revealed that both glomerular cross-sectional area (Fig. 5a) as well as glomerular volume (Fig. 5b) increased in diabetic rats in first month itself. There was no significant change in second and third month but increased further in the fourth month indicating that alterations are compounded as a result of constant exposure to hyperglycemia.

Correlation analyses—Correlation coefficient by linear regression analyses was carried out between various parameters (Fig. 6). An inverse correlation was observed between FBS and total kidney GAGs ($r=0.578$, $P<0.001$) (Fig. 6a) indicating that sustained increase in blood glucose will have an impact on one of the important ECM components of kidney, the GAGs. Direct correlation with increase in sustained hyperglycemia was observed between FBS and GAG excreted ($r=0.845$, $P<0.001$) (Fig. 6b). An inverse correlation was observed between kidney weight and kidney GAGs ($r=0.628$, $P<0.001$) (Fig. 6c). Inverse correlations were also observed for other parameters such as total kidney GAGs vs. GAGs excreted ($r = 0.763$, $P<0.001$) (Fig. 6d). Direct correlation with increase in sustained hyperglycemia was observed between FBS and type IV collagen excretion ($r=0.73$, $P<0.001$) (Fig. 6e). Thus all the above parameters tested with respect to kidney showed high degree of correlation.

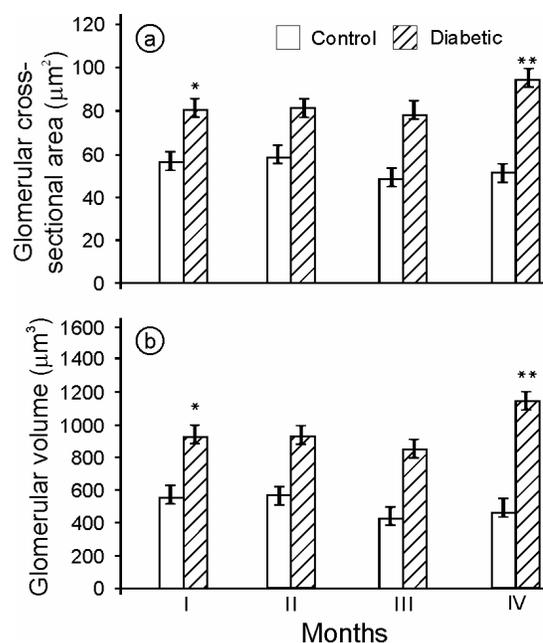


Fig. 5—Morphometric evaluation of kidney sections. PAS-stained kidney section from each rat was evaluated for glomerular cross-sectional area (a) and glomerular volume (b). Glomerular cross sectional area was measured using Image J (NIH). Values are represented as mean \pm SD of atleast 10 glomeruli from each rat. ***represent statistical comparison of I month control rats with I month diabetic rats and comparison between I month diabetic rats with II, III, IV month diabetic rats, respectively. A P value of ≤ 0.05 was deemed statistically significant.

Discussion

DN is one of the manifestations of secondary complications of diabetes. Kidney is the main target organ affected. In this communication, an attempt was made to determine systematically the progressive nature of early phase DN for which the changes in various basic and kidney-related parameters were looked into for 4 months duration.

Diabetes is characterized by sustained hyperglycemia, which causes changes in metabolic

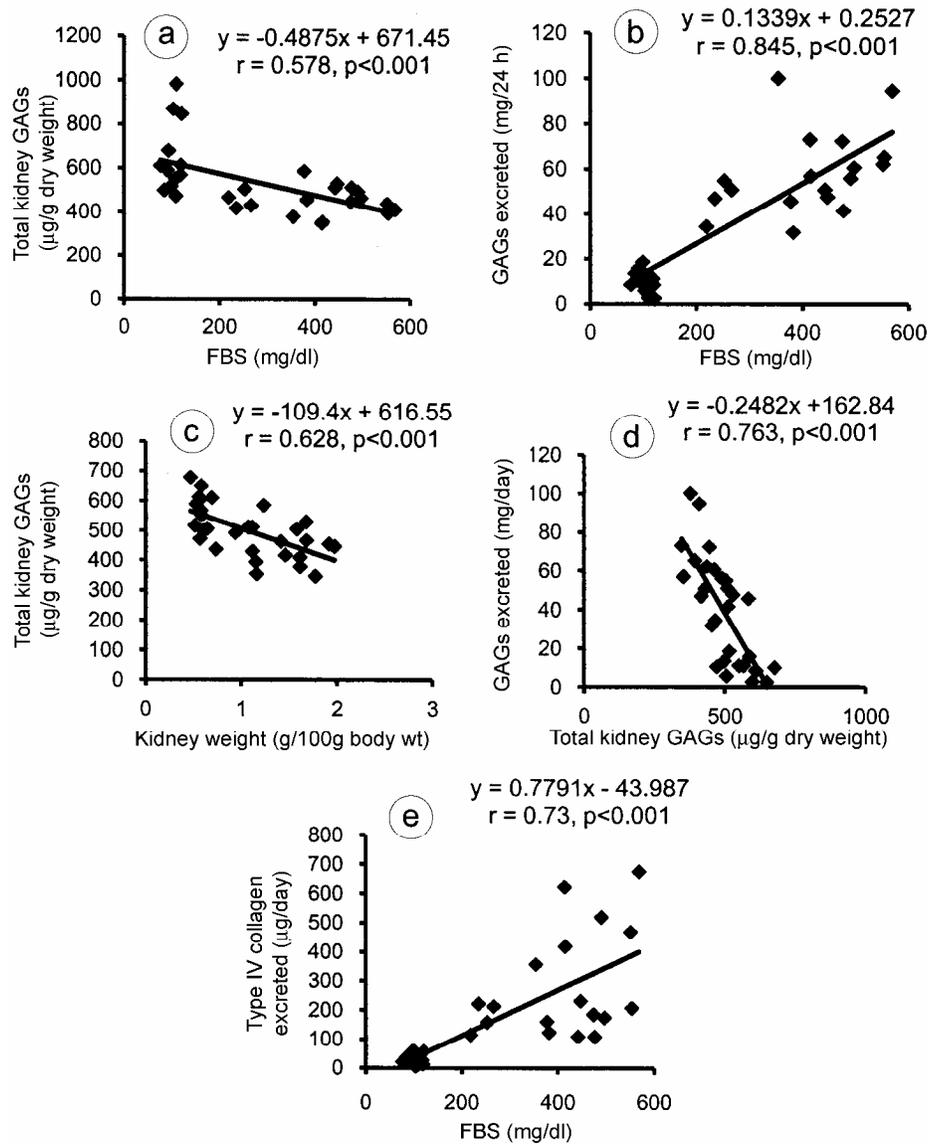


Fig. 6—Correlation analyses between various parameters - Correlation coefficient between various parameters were carried out by linear regression analysis. FBS vs. Kidney GAG (a), FBS vs. GAG excreted (b), Kidney weight vs. Kidney GAG (c), Kidney GAG vs. GAG excreted (d), and FBS vs. Type IV collagen excretion (e).

and hemodynamic properties¹⁸. Microalbuminuria is reported to occur after the onset of DN¹⁹. It is also one of the important markers which are routinely used to monitor DN. The results revealed that with the progression of duration of diabetic status, there is increased excretion of albumin, which was more pronounced at the end of 4 months.

Renal enlargement is one of the key features occurring during initial changes of diabetes²⁰. In earlier stages of DN, a hypertrophy and hyperfunction of the kidney with typical increase in kidney size and GFR was observed²¹. This is due to factors such as

glomerular hypertrophy and nephromegaly²². Whole kidney enlargement (nephromegaly), an early feature of both experimental and human diabetes occurs due to combination of tubular hypertrophy, hyperplasia and interstitial expansion²³. In the present study increased renal enlargement was observed, which was evident in first month. The condition was further aggravated at the end of four months. The degree of renal enlargement directly correlated with glycemic levels, which stresses the importance of glycemic control in the management of diabetes. Reversal of glomerular hyperfiltration and renal hypertrophy can

be accomplished by blood glucose normalization²⁴. Renal enlargement was validated by morphometric analysis.

Renal enlargement was marked by decreased amounts of GAGs in kidney and increased urinary excretion. GAGs are located as anionic sites in lamina rarae of GBM and mesangial matrix where they serve as charge selective barriers of filtration process²⁵. There was a progressive decrease with the increased duration of DN indicating the deleterious effect of sustained hyperglycemia on the kidney. It has been hypothesized that decreased GAG levels in GBM of kidney results in its leakiness leading to albuminuria²⁶. The decrease in GAG levels in the kidney could be due to decreased synthesis or increased degradation. In recent years increase in heparanase activity, an enzyme which is known to degrade GAGs such as heparan sulfate was found to increase in glomeruli of diabetic rats²⁷. An increase in urinary GAGs was observed during diabetes which negatively correlated with kidney GAGs. This could reflect on increased GAG degradation leading to the quantitative changes in kidney GAGs and its increased excretion in urine. Increased urinary excretion of GAGs was observed in diabetic nephropathic patients by other groups²⁸.

Another major ECM component of the kidney, type IV collagen, which is shown to increase during diabetes²⁹, was excreted in higher amounts. It suggests to increased degradation as a result of diabetic condition. Increased excretion of type IV collagen was observed in Db/Db mouse, an experimental model for genetic diabetes, which exhibits glomerular pathology³⁰. In the present study, the increase in type IV collagen excretion was more pronounced at the end of four months.

Histopathological evaluation revealed that hyperglycemia as a result of diabetic status was a trigger to increase glomerular cross sectional area as well as glomerular volume. Pathological changes were not very obvious in the first month of diabetic condition. However glomerulosclerosis was observed at the end of four months of sustained hyperglycemia. Various factors which contribute to glomerular enlargement include growth of glomerular cells and an increased accumulation of ECM³¹.

Correlation analyses revealed the direct correlation between glycemic status and parameters such as kidney weight, GAG and type IV collagen excretion but inverse correlation to kidney GAGs. Most of the

parameters looked into, aggravated at the end of four months of diabetic status indicating that sustained hyperglycemia would result in worsening of diabetic condition as a result of manifestation of diabetic complications.

Onset of DN in rats begins subtly after one month of diabetes but gets vitiated and more pronounced at the end of four months. Most of the parameters tested showed correlation to glycemic levels. Hence appropriate glycemic levels will result in lessening of diabetic complications.

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