Advanced Glycation End Products in Serum Predict Changes in the Kidney Morphology of Patients With Insulin-Dependent Diabetes Mellitus

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The biochemical mechanisms that cause the development and progression of diabetic nephropathy are unknown. Advanced glycation end products (AGEs) might play a role, as shown by increased levels of tissue-bound and circulating AGEs that correlate with the severity of diabetic nephropathy. The aim of the present study was to investigate if circulating AGEs predict the progression of morphological pathology in patients with diabetic nephropathy. We have developed an immunoassay to determine serum levels of AGEs. In a prospective clinical trial of young insulin-dependent diabetes mellitus (IDDM) patients with microalbuminuria, kidney biopsies were taken at baseline and after 24 to 36 months. The biopsies were analyzed for structural changes in the glomeruli by quantitative morphometry (electron microscopy). We have retrospectively analyzed serum AGEs. The mean serum level of AGEs at the start of the study was 18.7 U/mL (95% confidence interval [CI], 16.9 to 20.5). A positive correlation between serum AGE levels at the start of study and changes from baseline to follow-up study in basement membrane thickness ($r = .56$, $P < .02$) and matrix/glomerular volume fraction ($r = .57$, $P < .02$) was demonstrated. In a stepwise regression analysis with changes in the matrix/glomerular volume fraction as the dependent variable, serum AGE levels at the start of the study proved to be a significant independent variable ($P < .02$), whereas the mean hemoglobin A_{1c} (HbA_{1c}) or HbA_{1c} at the start was not. This study shows that serum AGEs predict the progression of early morphological kidney damage during 2.5 years in patients with IDDM.

LONG-TERM BLOOD LEVELS of glucose as measured by increased hemoglobin A_{1c} (HbA_{1c}) are the most important factor in the development and progression of early diabetic nephropathy.[1-4] Recently, we have shown an association between the mean HbA_{1c} and the progression of glomerular morphological changes in patients with diabetes and microalbuminuria.[5] However, the biochemical mechanisms behind these changes have not been established. In the Maillard reaction, glucose reacts nonenzymatically with proteins and forms covalently bonded Amadori products such as HbA_{1c} in vivo.[6] A small proportion of these products are modified into a heterogenous group of chemically and biologically active modified proteins called advanced glycation end products (AGEs).[7] Several cell types have been shown to contain specific receptors for AGE-modified proteins,[8,9] and the binding of AGEs to specific receptors stimulates cytokine[10] and growth factor[11] release and increases the synthesis of matrix proteins.[12] Recently, collagen-bound AGEs in skin were reportedly increased in diabetic patients with microalbuminuria.[13,14] Therefore, AGE-modified proteins may be part of the mechanism leading to development and progression of diabetic nephropathy.

The investigation of a possible relationship between AGEs and morphological pathology in diabetic nephropathy can be elucidated by the determination of serum levels of AGEs. Therefore, we have developed an assay for serum AGEs. In a retrospective longitudinal study in which serial kidney biopsies were taken at the start and after 24 to 36 months, we analyzed serum AGEs to determine if circulating AGEs can predict the progression of early diabetic glomerulopathy.

SUBJECTS AND METHODS

Subjects

Three hundred seventy-one patients with insulin-dependent diabetes mellitus (IDDM) between 10 and 30 years old with a diabetes duration of more than 5 years were screened for persistent microalbuminuria. Forty-five fulfilled the criterium, and 33 of these agreed to take part in a prospective study.[5,15] Three patients withdrew from the study. In the remaining group of 30 patients, those who were older than 18 years and who consented to a renal biopsy (n = 18) were included in a kidney morphology study[5,15] and represent the patients in the present study. AGEs, diabetes duration, and albumin excretion range (AER) did not differ between the 18 patients in the biopsy study and the remaining 12 who took part in the prospective study. Persistent microalbuminuria was defined as an AER between 15 and 200 μg/min in at least two out of three overnight urine samples taken during 1 year. Patients provided written consent after having received thorough information. The protocol was approved by the regional Ethics Committee.

Clinical characteristics of the study group are shown in Table 1. None of the patients had proliferative retinopathy. One patient had blood pressure levels higher than 140/90 mm Hg. Although initially hypertensive (150/98 mm Hg), this patient's blood pressure declined to 145/85 mm Hg after 6 months without antihypertensive treatment.

All patients studied in the present report have been included in previous studies of diabetic glomerulopathy.[5,15] In the original study of 18 patients with microalbuminuria, the aim was to clarify the effect of mean blood glucose levels on the progression of morphological changes at an early stage of diabetic nephropathy. To determine possible differences in the mean blood glucose concentration, patients were randomized in the priority, mean 1-year prestudy HbA_{1c}, duration of diabetes, median 1-year prestudy AER, age, and sex, to receive either continuous subcutaneous insulin injection (CSII) by a portable insulin pump or conventional treatment (CT) multiple injections or two to three injections per day. In the present investigation, we studied whether the concentration of serum AGEs at the start of the study was a predictive factor for the progression of early diabetic glomerulopathy. CSII and CT groups were combined because the mode of administering insulin per se

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Table 1. Clinical Characteristics of 18 IDDM Patients (mean and 95% CI)

Characteristic	Baseline		24 to 36 Months	
	Mean	95% CI	Mean	95% CI
Age (yr)	20	18-21		
Duration (yr)	11	10-13		
HbA_{1c} (%)	10.1	9.2-11.1	9.2	8.5-9.8*
AER (μg/min)†	25.8	20.5-53	20.5	14.0-109
Blood pressure (mm Hg)				
Systolic	125	119-131	125	118-132
Diastolic	81	77-85	83	79-86
GFR (mL · min^{-1} (1.73 m^2)$^{-2}$)	143	130-156	150	135-165

*P = .03 (paired Student's t test).
†Median value (mean of 2 measurements on each patient).

is not thought to influence the progression of early diabetic nephropathy.[16]

Methods and Reagents

The production of reagents was achieved according to the methods used by Makita et al.[17] Briefly, AGE-modified bovine serum albumin (AGE-BSA) and AGE-modified bovine pancreatic ribonuclease (AGE-RNase) were obtained by incubating the proteins (25 to 50 mg/mL) in 500 mmol/L phosphate buffer, pH 7.40, containing 500 mmol/L glucose for 12 weeks at 37°C. Two female New Zealand white rabbits received four primary immunizations and one booster immunization of AGE-RNase emulsified in Freund complete adjuvant. Specificity of the antisera was examined by the competitive enzyme-linked immunosorbent assay system. Anti-AGE antibodies detected AGEs that form in vivo such as AGE-collagen,[17] AGE-hemoglobin,[18] and AGE–low-density lipoprotein (LDL),[19] as well as AGEs made in vitro, such as glucose-derived AGE-RNase, glucose-derived AGE-albumin, glucose-derived AGE-LDL, and glucose-derived AGE–collagen IV.[17,18] Different glycosylating sugars such as glucose, glucose-6-phosphate, and fructose, when incubated with albumin, also produce an antigenically cross-reactive epitope. However, the antisera do not recognize unmodified RNase, albumin, hemoglobin, LDL, acetyl LDL, or collagen IV.[17]

The anti-AGE antibodies used in the assay were identical to the ones described previously.[17]

Fluorescence Immunoassay

Twelve-well microtiter strips (catalog no. 1244-550; Pharmacia, Uppsala, Sweden) were coated with 0.1 mL AGE-BSA (25 μg/mL) diluted in 0.05 mol/L carbonate buffer, pH 9.8, and incubated overnight with shaking at room temperature. The strips were covered and stored at 4°C with coating buffer. Immediately before the assay, the wells were washed six times in DELFIA washing buffer (catalog no. B117-100; Wallac, Turku, Finland). One hundred microliters AGE-BSA standard (in triplicate) or threefold-diluted serum sample (in duplicate) diluted in assay buffer (DELFIA assay buffer no. 1244-106; Wallac) was added to each well together with 50 μL anti-AGE antiserum diluted 1,000-fold in assay buffer. For the assay, six standard solutions consisting of 0, 0.75, 1.5, 3, 6, 12, and 24 μg/mL AGE-BSA standard were used. The strips were incubated with shaking at room temperature for 2 hours and then washed six times in washing buffer. After washing, 100 μL per well of Europium-labeled anti–rabbit immunoglobulin G antibodies (catalog no. 1244-100; Wallac) at a final concentration of 0.1 μg/mL in assay buffer were added. The strips were incubated with shaking for 1 hour and washed six times. Finally, the strips were incubated with DELFIA Enhancement solution (catalog no. 1244-105; Pharmacia), with shaking for 5 minutes at room temperature, and the Europium ion chelate specific fluorescence was measured in a 1232 DELFIA Fluorometer (Wallac).

AGE Units

One AGE unit was defined according to Makita et al[17] as the competetive activity of 1 μg AGE-BSA standard. Final serum concentrations of AGEs were corrected for total protein concentration in each serum sample in the following equation: (AGE, U/mL) × (sample protein concentration/mean protein concentration of all sera measured).

Instrumentation

Europium fluorescence was measured by a plate fluorometer (1232 DELFIA Fluorometer; Wallac).

Other Analyses

The total protein concentration in each serum sample was determined by the Biuret method (Boehringer, Mannheim, Germany), which has a coefficient of variation (CV) less than 2%. HbA_{1c} was analyzed with a high-performance liquid chromatography method (Diamat analyzer; Biorad, Richmond, CA). The normal range was 4.3% to 6.1%, with an interassay CV of 3%. AER was measured in three timed overnight urine samples 1 year before the first biopsy and then at 2-month intervals. Albumin concentration was measured by immunoturbidimetry in samples kept at 4°C from 1 to 3 days. The interassay CV was 4.7% in the range of 10 to 50 mg/L.

The glomerular filtration rate (GFR) was measured by inulin clearance (Inulin; Laevosan, Linz, Austria) after oral water loading. High concentrations of interfering glucose were removed by glucose oxidase.

Blood pressure was measured by a conventional mercury sphygmomanometer with the patients sitting after a 10-minute rest. Diastolic pressure was recorded after the disappearance of Korotkoff's V sound.

Renal Biopsies

Ultrasound-guided kidney biopsies were performed at entry and after 26 to 34 months.

Measurements of Glomerular Structural Parameters

Three glomeruli from each biopsy were sampled independently of size and structure, and thin sections were cut at three levels with a 60-μm interval. From all nine profiles in each biopsy, photomontages were produced at a magnification of 2,350× for the estimation of mesangial volume fraction per glomerulus by point counting. From the largest profile in each glomerulus, a subset of the area was photographed at 9,900× magnification for determination of the basement membrane thickness and the volume fraction of matrix per mesangium. By combining measurements at high and low magnification, the volume fraction of matrix/glomerulus was derived. The patients had an increased matrix/glomerular volume fraction compared with nondiabetic controls, as reported previously. For details, see Bangstad et al.[15]

Serum Samples

Serum samples collected at the start of study and after 12 and 24 to 36 months were immediately frozen at −70°C (up to 4.5 years) until analysis.

Determination of Molecular Weight Fractions Identified by the AGE Immunoassay

The Superdex 75 prepacked in HR 10/30 column (1 × 30 cm) connected to the FPLC system (Pharmacia) was used for gel filtration studies. The samples (200 μL undiluted serum) were eluted with 0.05 mol/L phosphate buffer, pH 7.4, with 0.15 mol/L NaCl. The flow rate was 0.5 mL/min, and fractions of 0.5 mL were collected in tubes containing 0.1 mL 0.1% human serum albumin. AGE levels were measured in all fractions. Serum samples from one normal control and

one diabetic patient with and one without microalbuminuria were studied by gel filtration.

Statistical Analysis

Changes over time in glomerular morphology were analyzed by two-tailed Student's *t* test (paired analysis). A stepwise linear regression analysis was used to study the effects of serum levels of AGEs and HbA_{1c} on changes in glomerular morphology. Linear correlations were tested by least-squares regression to the mean or Spearman rank-order correlation. Results are presented as the mean with a 95% confidence interval (CI), or as the median with CI when the data did not show a normal distribution. The level of significance was set at P less than .05. Calculations were performed using the Number Cruncher Statistical System (Kaysville, UT).

RESULTS

Immunoassay

Serum levels of AGEs were measured in duplicate on two separate occasions to calculate the interassay CV (9.7%) and the intraassay CV (8.7%) within the operating range (3 to 6 U/mL). The mean lowest detectable concentration of AGE-BSA, defined as twice the standard deviation of the maximum binding, was 0.5 U/mL. This is well below the actual operating range between 3 and 6 U/mL (Fig 1). A dilution curve for a serum sample was nearly parallel to the standard curve (Fig 1).

Clinical Parameters

Using paired statistics, there were no significant differences in the clinical parameters described in Table 1 from baseline to follow-up study, except for HbA_{1c}, which decreased from a mean of 10.1% (95% CI, 9.2% to 11.1%) at baseline to 9.4% (8.4% to 10.4%) after 12 months ($P < .03$ *v* baseline) and 9.2% (8.5% to 9.8%) after 24 to 36 months ($P < 0.01$ *v* baseline). Mean HbA_{1c} during the study was 9.3% (8.6% to 10.1%).

Serum Levels of AGEs

Baseline serum levels of AGEs (mean of duplicates) were 18.7 (16.9 to 20.5) U/mL. These concentrations correlated significantly with the corresponding levels of HbA_{1c} ($r = .56$, $P < .02$), but not with age, duration, AER, blood pressure, or GFR. Serum AGE values after 12 and 24 to 36 months were 17.6 (16.5 to 18.8) U/mL and 16.8 (15.2 to 18.4) U/mL, respectively (NS). No significant differences were seen in serum AGE values between the original CSII and CT groups.

Determination of Molecular Weight Fractions Identified by AGE Immunoassay

Greater than 90% of AGE activity was found in the fractions corresponding to the molecular weight range of 50 to 170 kd. Minor activity was seen within the 1.3- to 2.0-kd range. No differences in the molecular weight distribution of AGEs were detected between the sera tested.

Glomerular Morphology

A significant increase in the basement membrane thickness from baseline to follow-up after 26 to 34 months was observed: 593 (545 to 642) nm versus 691 (622 to 761) nm ($P < .02$). The matrix/glomerular volume fraction did not change significantly during the study in the group analysis (0.13 (0.12 to 0.14) *v* 0.13 (0.12 to 0.14), NS).

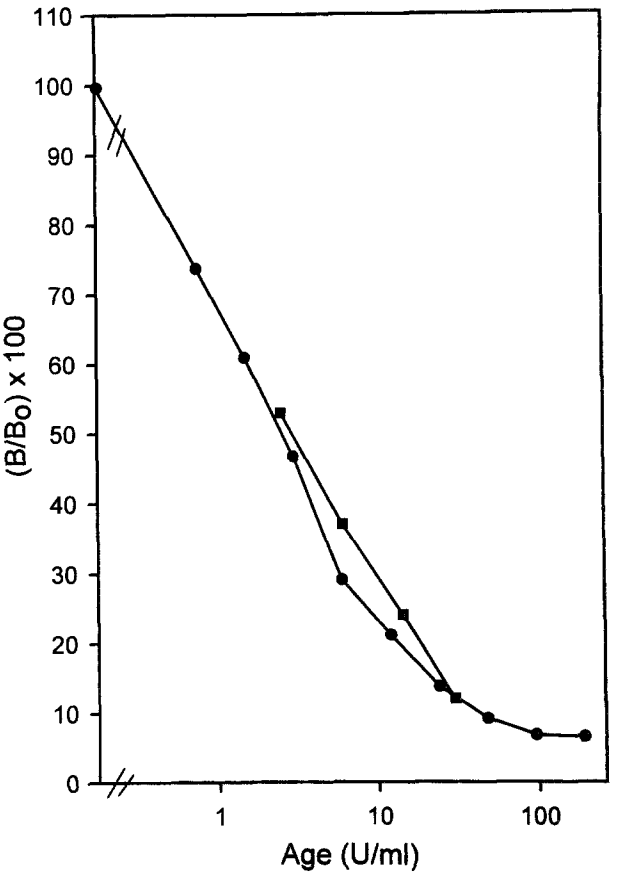

Fig 1. Typical competition curve for the AGE assay. In this competitive fluorometric immunoassay, AGE-BSA or serum AGE competed with AGE-BSA immobilized in the tube wall for binding of anti-AGE antibodies. Thus, binding of anti-AGE antibodies to immobilized AGE-BSA would decrease at increasing concentrations of AGEs in solution, ie, serum AGE (■) or AGE-BSA standard (●). B_0 is defined as the fluorescence count without added AGE-BSA standard. B represents fluorescence in the presence of added AGE-BSA from standard or sample (mean of triplicate determinations). Results for a serum sample analyzed undiluted or diluted twofold, fourfold, or eightfold are also shown (■).

Serum AGEs and HbA_{1c} Versus Changes in Glomerular Morphology

Serum levels of AGEs at the start of study showed a positive correlation with the changes from baseline to follow-up study in basement membrane thickness ($r = .56$, $P < .02$; Fig 2) and matrix/glomerular volume fraction ($r = .57$, $P < .02$; Fig 3). There was also a correlation between mean serum AGEs during the study and change in basement membrane thickness ($r = .58$, $P < .02$). No cross-sectional correlations were found between kidney morphology and serum levels of AGEs or HbA_{1c} at the start of study or after 24 to 36 months. But serum AGEs at baseline correlated significantly with matrix/glomerular volume fraction after 24 to 36 months ($r = .48$, $P < .04$). The correlation between serum AGEs at baseline and basement membrane thickness at the end of the study showed a positive trend ($r = .46$, $P < .06$). In an analysis separating the two intention-

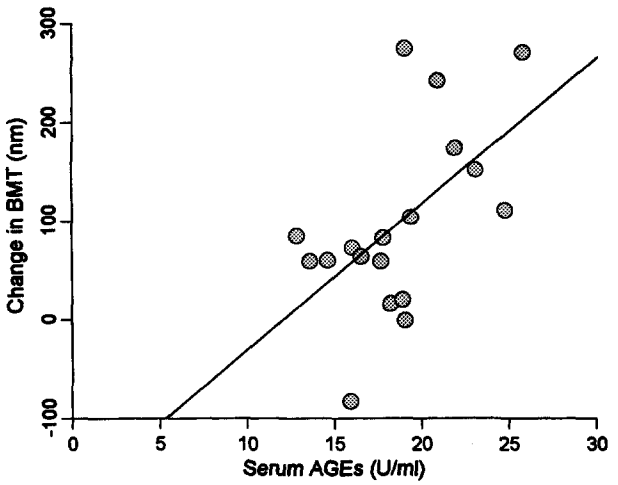

Fig 2. Changes in basement membrane thickness (BMT) during 24 to 36 months *v* initial serum AGEs in 18 microalbuminuric IDDM subjects ($r = .56$, $P < .02$).

to-treat groups, CSII and CT, there were no significant differences concerning serum AGEs at the start or mean AGEs and changes in kidney morphology.

HbA_{1c} at the start of the study correlated with changes in basement membrane thickness ($r = .64$, $P < .01$) but not with changes in matrix/glomerular volume fraction ($r = .24$, NS). There was, as shown previously,[5] a strong association between mean HbA_{1c} over the study and changes in basement membrane thickness ($r = .70$, $P < .01$). Mean HbA_{1c} or mean AGEs did not correlate significantly with changes in matrix/glomerular volume fraction ($r = .31$ and $r = .33$, respectively).

In a stepwise regression analysis with serum AGEs and HbA_{1c} at the start and mean serum AGEs and mean HbA_{1c} as independent variables, serum AGEs at the start was found to be an independent factor regarding changes in matrix/glomerular volume fraction ($P < .02$). For changes in basement membrane thickness, mean HbA_{1c} and mean serum AGEs were independent factors ($P < .0005$ and $P < .02$, respectively).

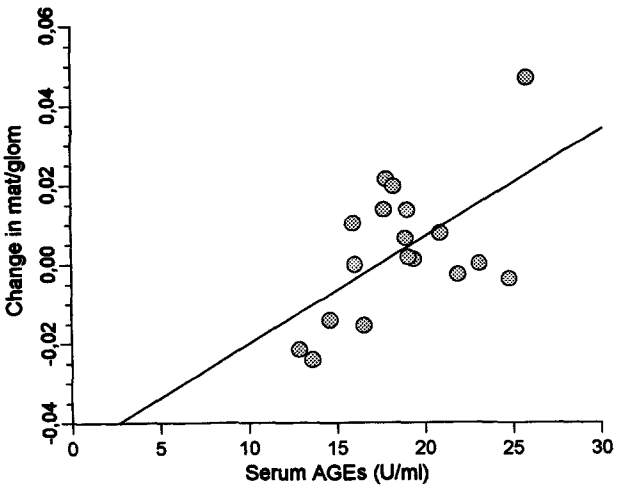

Fig 3. Changes in the matrix/glomerular volume fraction (mat/glom) during 24 to 36 months *v* initial serum levels of AGEs in 18 microalbuminuric IDDM subjects ($r = .57$, $P < .02$).

DISCUSSION

In this study, we have shown a relationship between serum levels of AGEs and the progression of morphological pathology in the glomeruli of diabetic patients with microalbuminuria. Our results suggest a possible pathogenetic role for AGEs in the progression of early diabetic nephropathy.

A close relationship between AGEs in serum and AGE-BSA made in vitro was indicated by the serum dilution curve, which was parallel to the standard curve (Fig 1). This indicated that serum AGEs and AGE-BSA share common antigenic sites.[17,20,21]

The present immunoassay had satisfactory reproducibility and sensitivity. Serum dilutions indicate that the method has an acceptable accuracy.

Serum AGEs were high–molecular-weight substances probably due to the fact that serum proteins are high–molecular-weight substances that are able to form AGEs. Others have found that 80% of serum AGEs in diabetic patients with kidney failure are low–molecular-weight peptides[21] or that low–molecular-weight AGEs are increased in kidney failure.[22] This might be due to a reduced clearance of low–molecular-weight fractions in kidney failure not observed in patients with normal kidney function as shown by a normal or supranormal GFR in our study group.

It was previously shown that the levels of collagen-bound AGEs in skin biopsies using the same antibodies correlated with a stepwise increase in diabetic patients with normoalbuminuria, microalbuminuria, and macroalbuminuria, respectively.[14] Skin collagen levels of pentosidine and carboxymethyllysine (CML) were also elevated in diabetic patients with microalbuminuria or macroalbuminuria.[13,23] AGEs in skin collagen might represent long-term tissue deposition of pathogenic compounds in long-lived proteins. Our findings may indicate that circulating AGEs reflect the process of advanced glycation taking place in the mesangial cell.[24] Alternatively, this may be caused by an increased matrix production due to binding of AGEs to specific receptors on the mesangial cell.[12]

In a stepwise regression analysis, mean serum levels of AGEs predicted changes in matrix/glomerular volume fraction, whereas the HbA_{1c} level at the start and mean HbA_{1c} over the study period did not. This may suggest that late glycation, ie, AGEs, influence the expansion of the mesangial matrix, which is important since mesangial matrix expansion is the dominant change in advanced stages of glomerulopathy. This theory is supported by the findings of Soulis-Liparota et al.[25] They have shown that inhibiting the formation of AGEs with aminoguanidine in streptozotocin-diabetic rats prevents mesangial expansion, but does not influence basement membrane expansion. Nevertheless, our present findings need to be interpreted with caution, bearing in mind that the number of patients was small and the fact that the matrix/glomerular volume fraction did not change when considering the patients as a group.

The lack of cross-sectional associations between serum AGEs and kidney morphology at the start and end of the study is possibly due to that the production of AGEs precedes morphological changes. The same was found regarding HbA_{1c}. Serum

AGEs at the start of the study might reflect previous AGE levels. This could explain the higher association between changes in kidney morphology and serum AGEs at baseline compared with mean serum AGEs.

Support for a possible causal role of AGEs in the development of diabetic nephropathy was recently obtained by findings in normal rats. Administration of AGE-modified albumin caused a 50% increase in renal tissue levels of AGEs, basement membrane widening, and mesangial matrix expansion.[26] Our hypothesis is further supported by the finding that AGEs are present in human diabetic kidneys as shown by immunohistochemical staining of anti-AGE antibodies primarily in the vascular intima of kidney arteries, but also within nodular and severe diffuse lesions of glomeruli and in hyaline deposits of renal arterioles.[27]

The present study is the first to link measurements of AGEs in vivo with diabetic renal pathology. It suggests a role for AGEs in the progression of early morphological kidney damage in diabetes. These data need to be confirmed by prospective intervention studies with inhibitors of AGEs formation. One potential agent in this regard is aminoguanidine, which has been shown to prevent the mesangial expansion and attenuate the increase in urinary albumin excretion and AGE-related fluorescence in the glomeruli of diabetic rats.[25]

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