PROTECTIVE EFFECT OF INSULIN TREATMENT ON EARLY RENAL CHANGES IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Abstract

Objectives. Chronic kidney disease is a progressive complication of diabetes mellitus (DM). This study aimed to analyse early renal changes in streptozotocin induced diabetic rats and demonstrate the effect of early treatment with insulin on kidney's histology.

Methods. 30 male-adult Sprague-Dawley rats were included in the study. Diabetes was induced in 24 of the rats by a single injection of 65 mg/kg streptozotocin dissolved in saline. 5 units/day NPH insulin injection was started to 10 rats as treatment group and 11 rats were followed untreated. 6 rats constituted the control group. Induction of diabetes failed in 3 animals and 3 untreated rats died during the study. After 21 days, all rats were sacrificed and their kidneys were removed to obtain histological sections to be evaluated by light microscopy.

Results. Ten treated and 8 untreated diabetic rats and 6 healthy controls, totally 24 rats completed the study. There was a significant weight loss in treated and untreated diabetic groups and a weight gain in the control group ($p < 0.05$). Final blood glucose levels were significantly higher in untreated diabetic group when compared to treated diabetic and control groups and higher in treated diabetic group when compared to control group. Histological analysis of kidney sections showed normal morphology in control group. Changes like increased mesangium, tubular atrophy and tubules with dilated lumen and irregular cell shapes were found in the untreated group whereas, glomerulus and mesangium showed similar morphology with control group with a few changes in tubules, in insulin-treated group.

Conclusion. In DM, renal changes start at an early point and it is possible to prevent/delay those changes at this point with early intervention of insulin treatment.

Key words: Diabetes mellitus, chronic kidney disease, streptozotocin-induced diabetic rats, diabetic nephropathy.

INTRODUCTION

Diabetes mellitus (DM) is the most common cause of chronic kidney disease. Diabetic nephropathy

occurs in 20–40 % of patients with DM and it is one of the leading causes of morbidity and mortality in DM patients. Considering globally increasing prevalence of diabetes, it is also expected to have an increasing number of chronic kidney diseases (CKD) related to diabetes (1-3). Screening for CKD in DM and using approved tools urgently to delay/prevent its progression is of great importance. The most effective way to prevent the progression to CKD in DM is to achieve a better glucose control. Insulin is the most effective glucose lowering agent and it is known that glucose regulation by insulin treatment decreases the risk of microvascular complications (2,4-5).

Studies performing analysis of early renal changes in DM and the impact of treatment on these changes are limited. Rats have been the species of choice for disease modelling over many decades. Rat models of hypertensive renal damage, diabetic nephropathy, acute/chronic kidney disease and renal transplantation contributed widely to our understanding of renal diseases. Diabetic animal models have also gained popularity because of the similarities to human diabetes. Rats used with experimental purposes are able to mimic most of the features of human renal changes related to diabetes including progressive proteinuria, key pathological findings like glomerulosclerosis and tubulointerstitial fibrosis, and the activation of several pathways that play a role in human diabetic nephropathy. An ideal study model can be created with application of insulin regimen to a diabetic rat model (6-7). Understanding the key aspects of renal pathological changes in diabetes with such a model can help to identify new targets for treatment.

This study aimed to analyse early renal changes in streptozotocin induced diabetic rats and demonstrate the effect of early treatment with insulin on kidney's histology.

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ANIMALS AND METHODS

Thirty male adult Sprague-Dawley rats weighing 210-330 g were included in the study. All of them were grown in Experimental Medicine Research Institute (DETAE) for the study. After 12 hours of fasting, 24 of the rats were given a single intravenous injection of 65 mg/kg streptozotocin (STZ) dissolved in 0.9% NaCl into the tail vein to induce diabetes. The remaining 6 rats constituted the healthy control group. Following the injection, all animals were kept in equal environmental conditions and after 2 days, all rats injected with STZ were checked for the induction of diabetes by assessment of blood glucose levels in the tail vein using a glucometer (Rheamed®-Model No: SC-12A-AA, Sand County Biotechnology, Inc, Taiwan).

Animals with fasting blood glucose levels above 200 mg/dL were accepted as diabetic. Three rats were excluded from the study because diabetes was not achieved. Blood glucose levels of control group were also measured to confirm normoglycemia (60-100 mg/dL). Five units/day NPH insulin was started to 10 of the diabetic rats to form the treatment group and the remaining 11 diabetic rats were followed untreated. All animals were kept in standard rat cages with two animals in each cage, at $21\pm 2^{\circ}\text{C}$ and a 12/12 light/dark cycle. They were given standard rat pellet and water *ad libitum*. The body weight and blood glucose levels of all groups were monitored. All 3 groups were followed for 21 days in the same environmental conditions. Three rats from the untreated diabetic group died during the study, thus their data was excluded from the study group.

After 21 days, 24 rats (10 treated diabetic rats, 8 untreated diabetic rats and 6 healthy controls) that completed the study were sacrificed under ether anesthesia. Their abdomens were opened, their kidneys were removed with dissection and sections were obtained. Histological evaluation of sections was made with light microscopy for each group. Changes in blood glucose levels and body weight were evaluated using SPSS statistical analysis method.

Statistical method

Mean, standard deviation and median values were used for the statistics of explanatory variables. Distribution of variables was measured by Kolmogorov Smirnov test. ANOVA (Tukey test) was used for quantitative independent variable analysis. Paired samples t test was used for quantitative dependent variable analysis. SPSS 22.0 program was used for analysis.

Histological method

Kidney tissues were taken after sacrifice and fixed within 10% buffered formalin. Tissue samples were treated with increased ethanol series (70%, 90%, 96%, 100%) and embedded in paraffin. Paraffin sections (3-4 μm) were taken by microtome (Leica RM2255). Sections were stained with Periodic Acid Schiff (PAS) histologic stain and evaluated by Olympus BX40 light microscope. Image samples were taken using objectives 20x and 40x magnifications.

RESULTS

Ten treated diabetic rats, 8 untreated diabetic rats and 6 healthy control, totally 24 rats completed the study.

Mean body weight was 263.5 ± 32 gr initially and 220.8 ± 32 gr finally for treated group, 301 ± 17 gr initially and 243 ± 33 gr finally for untreated group, 289 ± 11 gr initially and 314 ± 10 gr finally for control group.

Mean blood glucose level was 413.5 ± 90 mg/dL initially and 218 ± 105 mg/dL finally for treated group, 289 ± 46 mg/dL initially and 408 ± 49 mg/dL finally for untreated group, 90 ± 7 mg/dL initially and 114 ± 20 mg/dL finally for control group. Initial (1st) and final (2nd) results for body weight and blood glucose levels are shown in Table 1 and Figures 1-2 .

Statistical analysis

There was a significant weight loss in treated and untreated diabetic groups ($p < 0.05$) and weight gain in control group at the end of study ($p < 0.05$). Weight change between initial and final measurements was significantly higher in treated and untreated diabetic groups when compared to control group ($p < 0.05$). There was no significant difference between

Figure 1. Body weight difference between treated diabetic, untreated diabetic and control groups.

Figure 2. Blood glucose (BG) difference between treated diabetic, untreated diabetic and control groups.

Figure 3. Control group kidney: a) General morphology of glomerulus and surrounding tubules (x20); b) Glomerular capillaries with normal features (x40); c) Proximal tubules with intact brush border (asterisk) (x40); d) Intact proximal and distal tubules (x40). G:glomerulus, c: capillaries T: tubules, P: proximal tubules, D: distal tubules, asterisk: brush border, arrow: Bowman capsule.

treated and untreated diabetic groups for weight change ($p > 0.05$) (Table 1).

In both treated and untreated diabetic groups, 1st blood glucose levels were significantly higher than in control group ($p < 0.05$). Second blood glucose levels were significantly higher in untreated diabetic group when compared to treated diabetic group and control group ($p < 0.05$). Second blood glucose levels were higher in treated diabetic group when compared to control group ($p < 0.05$).

In treated diabetic group, blood glucose levels decreased significantly at the 2nd measurement when compared to the 1st measurement ($p < 0.05$). In untreated diabetic group, blood glucose levels increased significantly at the 2nd measurement when compared to the 1st measurement ($p < 0.05$). In control group, blood glucose levels increased significantly at the 2nd measurement when compared to the 1st measurement ($p < 0.05$). As expected, decrease in BG levels between two measurements was significantly higher in treated diabetic group than in untreated diabetic group and control group ($p < 0.05$) and increase in BG levels between two measurements was significantly higher in untreated diabetic group than in control group ($p < 0.05$) (Table 1).

Histological analysis of the kidney sections

Control Group

In light microscopy, capillaries and mesangium of glomerulus showed normal morphology. Cells of proximal tubules had intact brush border with PAS positive stain on their apical membranes. Cells had normal morphology with their nucleus and cytoplasm. The lumen was clear. In distal tubules, basement membrane and the lumen were clear, intact nucleus and cytoplasm were detected. Collecting tubules showed normal morphology (Fig. 3).

Table 1. Body weight and blood glucose analysis of all groups

	Treated diabetic group		Untreated diabetic group		Control group		p
	Mean±s.d.	Median	Mean±s.d.	Median	Mean±s.d.	Median	
Weight							
1 st weight (g)	263.5 ± 32.4	259.5	301.1 ± 18.1*	306.0	289.8 ± 11.2	290.0	0.011 ^A
2 nd weight (g)	220.8 ± 32.3	215.5	243.4 ± 33.0	238.0	314.0 ± 10.9*†	313.5	0.000 ^A
Difference	-42.7 ± 19.0	-41.5	-57.8 ± 40.6	-57.5	24.2 ± 9.1*†	23.0	0.000 ^A
Intra Group p	0.000 ^P		0.005 ^P		0.001 ^P		
BG							
1 st BG (mg/dL)	413.5 ± 90.9	379.5	289.8 ± 46.0*	291.5	90.2 ± 7.9*†	91.0	0.000 ^A
2 nd BG (mg/dL)	239.5 ± 68.5	245.5	408.6 ± 49.6*	400.5	114.0 ± 22.8*†	110.5	0.000 ^A
Difference	-174.0 ± 101	-159.0	118.9 ± 73.7	131.5	23.8 ± 18.1*†	23.0	0.000 ^A
Intra Group p	0.000 ^P		0.003 ^P		0.003 ^P		

^A ANOVA (Tukey test) / ^P Paired samples t test.

* Difference with Treated diabetic group $p < 0.05$ / † Difference with Untreated diabetic group $p < 0.05$.

Figure 4. Untreated diabetic group kidney: a-b) General morphology of glomerulus and dilated, degenerated tubules (x20); c-d) Glomerulus with increased mesangium (asterisk) and degenerated cytoplasm of tubule cells (arrow) (x40); e) Vacuolated degenerated tubule cells (arrow) (x40); f) Hyperplasia in some tubules and hyaline droplets accumulation on cytoplasm (x40); g-h) Hyaline droplets (dashed arrow) were detected in the cytoplasm of cells and karyolysis in the nuclear material (k) (x40). G: glomerulus, HP: hyperplasia, k: karyolysis.

Untreated Diabetic Group

Glomerulus showed eosinophilic deposits in the extracellular mesangium which caused increasing of the mesangium. Distal and collecting tubules had a dilated lumen and irregular cell shapes. Eosinophilic hyaline deposits were detected in the cytoplasm of tubular cells. Tubular atrophy with nuclear karyolysis and vacuolization of cytoplasm were seen especially in cells of distal tubules. Proximal tubules showed intact brush border with PAS+ staining but in these tubules increased cellularity (hyperplasia) and increased cell size were also detected. No basement membrane thickening was present (Fig. 4a-b).

Treated Diabetic Group

In the treatment group, glomerulus showed similar features with control group. There were no eosinophilic deposits or increased mesangium. Distal tubules showed intact morphology, no atrophy was detected. But a few eosinophilic hyaline droplets accumulation were seen in cells of tubules. Some tubules showed dilated lumens similar to diabetic group. A few tubules showed cytoplasmic degeneration (Fig. 5).

DISCUSSION

In DM, the kidney and the liver is responsible for excess glucose production via gluconeogenesis. Glucose filtration and glucose reabsorption are other mechanisms of action of the kidney on glucose regulation (8). The key for progressing to CKD in DM is hyperglycemia. Hyperglycemia has cellular toxicity that leads to changes in cellular growth, gene and protein expression. It increases extracellular matrix and

growth factor, also oxidative and glycosylation products indirectly and TGF- β mRNA expression that causes cellular hypertrophy and collagenous transcription in proximal tubules. Thickening of glomerular basement membranes, vascular occlusion, increased permeability and glomerular mesangial matrix widening might occur (9). In our study, there was increasing of the mesangium in glomerulus; tubules had dilated lumen, irregular cell shapes and increased cellularity (hyperplasia) but there was no basement membrane thickening.

Macedo *et al.* tested acarbose, insulin and combination of these 2 drugs to search for the effect of

Figure 5. Insulin treatment group: a) General morphology of glomerulus and tubules (x20); b) Capillary and mesangial matrix of glomerulus has normal features, cells of distal tubules showed intact morphology (x40); c-d) Proximal tubules have normal appearance with intact brush border, hyaline droplet accumulation (dashed arrow) and degeneration of cytoplasm (arrow) were seen in some tubules (x40). G: glomerulus, D: distal tubules, P: proximal tubules.

metabolic control on mesangial enlargement (ME) of kidney in alloxan-induced diabetic rats. Diabetic rats had increased glycemia, weight loss and vacuolization of renal tubules was present histologically similar to our study. Mesangial enlargement was present and acarbose+insulin treated rats showed similar ME with normal rats. But in contrast with our study, in insulin treated group, mesangial enlargement was only partially prevented. Also, those changes were seen starting the 3rd month whereas we saw them after 3 weeks which indicated that they started at an earlier stage (10).

In our insulin treatment group, glomerulus showed features similar to the control group. Rasch, also observed that achieving glycemic control with insulin treatment prevented ME in streptozosin-induced diabetic rats (11).

Different studies with STZ-induced diabetic rats showed that insulin treatment delayed the progression to diabetic nephropathy. Although glomerular basement membrane thickening, mesangial expansion and proliferation were present in untreated diabetic group, no comparable changes were found in insulin-treated group in the study by Michael *et al.* (12) and renal atrophy and elevated Na⁺,K⁺-ATPase activity which is an important part of renal hypertrophy and hyperfunction regressed to normal levels after insulin treatment in the study by Ku *et al.* (13). Insulin deficiency activates the expression of IGF-1 and IGF-1R in renal mesangial cells and increases cell proliferation, fibronectin and collagen IV synthesis which contributes to diabetic nephropathy. Thus, early administration of insulin might prevent the overactivation of IGF-1/IGF-1R pathway and their blockage can create a treatment target for diabetic nephropathy (14).

Although no glucose lowering agent is known to be superior to any other agent about prevention of renal complications due to lack of head-to-head studies; renoprotective effect of glucose control is well established (15) and insulin is the most effective glucose lowering agent among all diabetes medications. It can reduce any degree of hyperglycemia to target levels, mimics the physiology best and is easy to titrate to avoid hypoglycaemia due to delayed renal clearance (5).

Renoprotective effects of different treatments in STZ-induced diabetic rats are found in several studies but most common treatment used in literature is insulin as it is in our study and better results are gained with better glycemic control (2,11,16-20).

In our study, NPH insulin was used to improve glycemia and accordingly, preserve kidney function. Different studies used different kinds of insulin like

long acting insulin, insulin implants or pectin insulin patches and got similar effects (6-7) which implicated achievement of normoglycemia as the key factor to prevent kidney damage caused by diabetes. This feature was also proved in a study using spontaneously diabetic Torii rats (21).

ADVANCE study (the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation) highlighted the outcomes of intensive glucose control in DM. Supporting the previous data, it was not effective on macrovascular events, but major microvascular event incidence was decreased, with a significant percentage attributed to the reduction of nephropathy rates (22).

Sustained high blood glucose compared with blood glucose fluctuations were found to be protective from serious pathological changes (23). Supporting this data, Malatiali *et al.* did not find glomerular hypertrophy but expanded mesangial matrix in their study where twice daily mix insulin treatment was used in STZ-induced diabetic rats. But still the treatment prevented hyperfiltration and proteinuria implying that structural and clinical findings did not necessarily comply with each other (24). The short duration of the study which was only one week could be the reason for absence of structural changes, but although our study was comparably short, significant structural changes were observed in kidneys after 3 weeks.

Glomerular hyperfiltration occurs with different mechanisms in different animal models. Insulin-treated subjects have an increased renal blood flow and non-treated subjects have a tubular event causing hyperfiltration (25). Also, renal tubulointerstitial changes are seen as early sign of renal damage in other STZ-induced rat studies, supporting our findings (16, 26).

A study of longer duration using different kinds of diabetic rat models showed severe pathological changes in kidney leading to diffuse glomerulosclerosis and nodular lesions but the renal damage started with structural changes similar to our study (27). Our results showed that those early changes could be prevented with early intervention before leading to severe, permanent damage as found in this study after 3 months.

In conclusion, this study argues that in DM, renal changes start at an earlier point than presented in most of the studies in literature and it is possible to prevent or delay those changes at this point with early intervention of insulin treatment. Even slightest decrease in rates of this most common diabetes-related microvascular complication or its progress to end-stage-

renal disease would decrease morbidity and mortality, improve quality of life, decrease health costs and contribute to public health. Thus, early glycemic control using insulin treatment when necessary in DM patients must be considered as preventive therapy as well as diabetes medication.

Conflict of interest

Author declares no conflicts of interest.

Compliance with Ethical Standards and Animal

Rights

This study was approved by Experimental Medicine Research Institute (DETAE) Ethical Committee. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution and Guide for the care and use of laboratory animals, Eighth edition (2011).

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