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Consistency of Rat model for diabetic nephropathy

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Abstract
Diabetic nephropathy (DNP) is a chronic kidney disease caused by diabetes that leads to end stage renal diseases. The disease is being diagnosed clinically by using microalbuminuria test, which is highly nonspecific. Several animal models have been developed to elucidate the pathophysiology of DNP and to identify innovative therapies for preventing the progression of nephropathy. The animals were either induced with type 1 or type 2 diabetes and were allowed to progress to nephropathy. Since DNP is a chronic disease, the most common challenge faced by several investigators is to maintain the animals in diabetic condition for a longer period of time. Although both mice and rat models are used for studying DNP, rats are generally preferred because of the longer life-span. The rat models generally used for type-2 diabetes induced nephropathy are Goto-Kakizaki and obese ZUCKER rats. At the age of 14 weeks, these rats spontaneously develop diabetes and if they are maintained in hyperglycemic state for a time span of 12 months, they progressed to DNP. However, nodular glomerulosclerosis and tubulointerstitial fibrosis were not observed in these rats, which are the most prominent pathological features observed in human DNP.

Keywords: Diabetic nephropathy, glycosuria and proteinuria.

1. Introduction
Tesch *et al.* [1] have found that STZ at a dose of 45, 55 and 60 mg/kg body weight has to be injected in Spontaneously Hypertensive rats (SHR), Sprague Dawley (SD) and Wistar rats respectively for inducing type-1 diabetes by damaging the pancreatic beta cells. The diabetic rats were allowed to progress to diabetic complications by maintaining them in hyperglycemic state (Tesch and Allen, 2007) [1]. Several investigators have used high dose of STZ (60 mg/kg body weight) to analyze the effect of various drugs on DNP in a shorter span of time (Tuncdemir and Ozturk, 2011; Makino *et al*., 2002) [2, 3]. However, high dose of STZ is found to have non-specific toxicity on the tubular cells of kidney. So the nephropathy induced using high dose of STZ is considered to be a superimposed result of hyperglycemia and nephrotoxicity (Tay *et al*., 2005; Kraynak *et al*., 1995) [4, 5]. Simultaneously, in few studies, albuminuria has been induced in Wistar rats even at a low dose (35 mg/kg body weight) of STZ. So there is a need for optimization of STZ dosage for inducing DNP in Wistar rats. Davis *et al.* have predicted that in order to prevent the animals from the nephrotoxicity of STZ, the animals have to be supplemented with long acting insulin injection. Thus the animals are maintained in a desirable blood glucose level of 300-600 mg/dl, inducing DNP. This intended us to investigate if insulin supplementation is crucial for inducing DNP in Wistar rats.

Diabetes
Diabetes mellitus is a metabolic disorder characterized by elevated blood glucose level. The body is unable to metabolize the glucose formed either because of lack of insulin or because the cells are unable to respond to the insulin produced. It is an insidious disease with no clinical symptom in the pre-diabetic stage and only when the disease progress to overt stage, it is characterized by hyperglycemia. The foremost symptoms that patients could identify themselves are the presence of increased thirst (polydypsia), increased urination (polyuria) and increased hunger (polyphagia). Diabetes is diagnosed clinically by measuring the blood glucose level and glycated hemoglobin content. Patients with a fasting plasma glucose level of $\geq$ 126 mg/dl and glycated hemoglobin content of $\geq$ 6.5% are considered diabetic. The major factors that contribute to the pathogenesis of diabetes include genetic, environmental (diet, stress and lifestyle) and immunogenic as well as the use of several drugs (glucocorticoids, β- adrenergic agonists and statins).

Types of Diabetes

The most common classes of diabetes are the type 1 and type 2 diabetes. Other forms of diabetes such as gestational, impaired glucose tolerance and diabetes related to malnutrition are relatively rare.

Type 1 Diabetes

It is also called as insulin dependent diabetes mellitus. It is an autoimmune disorder, in which there is loss of pancreatic beta cells, resulting in insulin deficiency. Approximately 10% of the diabetic cases are affected by type 1 diabetes. It is also termed as juvenile diabetes, because the onset of type 1 diabetes occurs at an early age, specifically affecting children. Patients with type 1 diabetes are supplemented daily with insulin in order to control the blood glucose level.

Type 2 Diabetes

It is also called as non-insulin dependent diabetes mellitus which accounts for more than 90% of the diabetic cases. It is often associated with obesity in which the cells do not respond to the insulin action. Type 2 diabetes was believed to have its onset only after 40 years, but recent studies have diagnosed it in young people too. Insulin administration is not mandatory in type 2 diabetic patients for survival, but they may become insulin dependent at advanced stages of the disease.

Diabetic Complications

Diabetic patients are prone to several acute complications such as diabetic ketoacidosis, hyperosmolar state, diabetic coma and periodontal diseases. Patients with uncontrolled blood glucose level i.e. prolonged hyperglycemia encounter deleterious effects in various organs such as eye, foot, skin, nervous system, kidney and heart. Because of the hyperglycemic condition, the endothelial cells lining the blood vessels take up more glucose through the insulin independent pathway. This results in the formation of more glycoproteins which lead to basement membrane thickening, thus affecting the blood vessels. When there is damage to the small blood vessels, they are grouped as microvascular complications and when the arteries are damaged, they are grouped as macrovascular complications. The macrovascular complications include coronary artery disease leading to heart attack, stroke and peripheral vascular disease. Atherosclerosis is considered as a major contributor for various cardiovascular diseases in diabetic patients. The major microvascular complications are;

Retinopathy*:* It is the damage to the retina of the eyes and is considered as the most common complications of diabetes. The chance of becoming blind is almost 25 times higher in diabetic patients compared to normal. The symptoms associated with retinopathy are loss of pericytes, venous dilations, endothelial cell proliferation with hyalinization and increased refractive power of lens. In most cases, damage to the eyes occurs in 5-10 years from the onset of diabetes.

Neuropathy*:* It is the damage to the nerves that leads to foot ulceration and amputation. The frequency of neuropathy ranges between 5-60% depending on the patient's age and duration of diabetes. The symptoms associated with neuropathy are non-painful numbness of the toe and foot, burning pain, fullness of the skin of the toes, feet and legs, dysesthesia, fasciculation, difficulty in swallowing and speech impairment. Nephropathy: It is the damage to the blood vessels and nephrons in the kidney that leads to renal failure. The kidney damage is predicted to occur between 15-25 years from the onset of diabetes. In type-1 diabetes, the progression to diabetic nephropathy is well defined, but in type-2 diabetes, it is less known because of the cardiovascular diseases associated with it.

Diabetic Nephropathy

Diabetic nephropathy (DNP) was first discovered by Clifford Wilson, a British physician (1906-1977) and Paul Kimmelstiel, an American physician (1900-1970) and the description of the disease was first published in 1936. Diabetic Nephropathy, also known as Kimmelstiel-Wilson syndrome is a chronic kidney disease caused by diabetes that leads to End Stage Renal Disease (ESRD). Though the pathophysiology of type-1 and type-2 diabetes is different, both the types of diabetes have an equal chance for progressing to nephropathy. It is predicted that 25-40% of the diabetic patients eventually progress to nephropathy within a time span of 15-25 years from the diagnosis of diabetes. The mortality in diabetic patients with nephropathy is thirty times higher than patients without nephropathy. Diabetic patients with poor blood pressure control and high cholesterol level are at high risk for progressing to nephropathy.

Symptoms of Diabetic Nephropathy

In the early stages of DNP, the disease shows no symptoms. Only at the later stages symptoms appear, as a result of excretion of high amount of protein in the urine. The initial histological changes in kidney include mesangial expansion, thickening of the glomerular basement membrane and podocyte loss. These changes further progresses to glomerulosclerosis and tubulointerstitial fibrosis in the final stages of DNP. Though several animal models have been established for DNP, they failed to develop glomerulosclerosis and tubulointerstitial fibrosis which are observed in the final stages of human DNP. So there is a need for standardization of rat model for diabetic nephropathy.

Results

Table-1: Optimization of STZ dosage

After 3 months	Dose of STZ (mg/kg body weight)		
	35	**45**	**60**
Mortality (%)	0	8.4	50
% of rats with blood glucose (>350 mg/dl)	33.3	90.9	100

The values represent the percentage mortality and rats with blood glucose of more than 350 mg/dl at the end of three months for different doses of Streptozotocin. In this preliminary study, the mortality rate was found to be very high (50%) in rats that were induced with 60 mg/kg body weight of STZ. This might have made various investigators to restrict their study period to 1-3 months. Simultaneously, in rats that were induced with a low dose of STZ i.e. 35 mg/kg body weight, the percentage of rats with hyperglycemia is only 33.3 though there is no mortality. However, more than 90% of the rats were maintained in hyperglycemic state with only 8.4% mortality (Table 2.1), when the rats were induced with 45 mg/kg body of STZ. This showed that 45 mg/kg body weight of STZ is more appropriate for maintaining the hyperglycemic state with less mortality, thus inducing chronic diabetic nephropathy in rats.

Table 2: Effect of insulin supplementation on urine biochemistry

Urinary parameters	Month 4			Month 7		
	Con	STZ	STZ + Ins	Con	STZ	STZ + Ins
Volume (ml/12 h)	3.2 ± 1.4	24.9 ± 2.2a	14.9 ± 0.8b,*	4.3 ± 0.8	24.7 ± 1.3a	18 ± 1.3b,n
Albumin (mg/24 h)	5.4 ± 1.3	38.9 ± 2.2a	23.1 ± 1.6c,*	4.8 ± 0.4	40 ± 2.4a	32 ± 2.2b,n
Creatinine (mg/dl)	1.4 ± 0.07	0.3 ± 0.01a	0.6 ± 0.02a,**	1.3 ± 0.1	0.2 ± 0.03a	0.4 ± 0.03a,n
Urea nitrogen (mg/dl)	9.6 ± 0.6	2.7 ± 0.3a	3.9 ± 0.4a,**	9.5 ± 0.8	2.8 ± 0.2a	3.6 ± 0.3a,n

Values represent the mean ± standard deviation of the samples (n=4). Significant difference between Con Vs STZ groups and Con Vs STZ + Ins groups: c $p< 0.05$; b $p< 0.01$; a $p< 0.001$. Significant difference between STZ and Ins: * $p< 0.05$; ** $p< 0.01$.

Pathological Changes in Diabetic Rats Progressing To Dnp

On kidney histological observation, the diabetic rats showed evidence for glomerular basement membrane thickening, mesangial expansion and proliferation in more than 25% of the glomeruli at the end of 20 weeks. But there was no evidence for nodular glomerulosclerosis. Tubules showed evidence for protein re-absorption droplets which confirmed proteinuria and glycosuria in 25% of the glomeruli. On the other hand, the changes were not comparable in diabetic rats treated with insulin. At the end of 28 weeks, the diabetic rats revealed evidence for nodular glomerulosclerosis in the mesangium. Mesangial nodules were also found to be PAS positive and the Masson Trichrome stain confirmed that the mesangial nodules were not comprised of collagen. Tubules showed evidence for proteinuria and glycosuria in more than 50% of the glomeruli. Hyaline arteriolosclerosis was also observed in the blood vessels. However, interstitial fibrosis was not evident even at the end of the study. In diabetic rats that were treated with insulin, though there was evidence for mild mesangial expansion, well-formed PAS positive mesangial nodules were conspicuously absent with no change in glomerular basement membrane. The tubules revealed evidence for glycosuria and protein re-absorption droplets in 25% of the cortical surface examined.

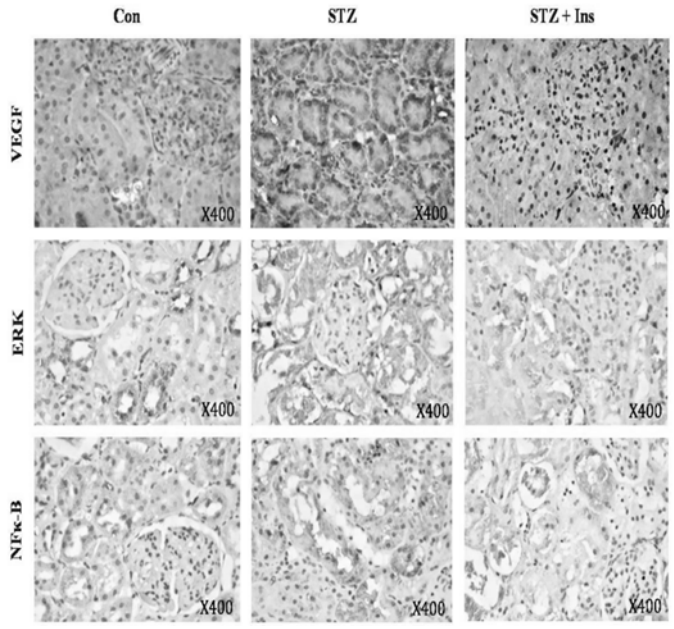

Fig 1: Immunohistochemical observation of VEGF, ERK and NF-κB.

Figure showing the immunohistochemical expression of VEGF, ERK and NF-κB in control (Con), diabetic (STZ), diabetic rats treated with insulin (STZ + Ins) at the end of 28 weeks (Magnification x400).

Discussion

In our study, the changes in urine biochemistry such as albumin, creatinine and urea nitrogen content were observed at the end of every month after the induction of diabetes. It was found that the urinary biochemical changes observed at the end of fourth month in diabetic rats were observed only at the end of seven months in diabetic rats supplemented with insulin. As speculated, insulin supplementation delayed the progression of nephropathy. This was further substantiated by the histological and immunohistochemical examinations of the kidney. At the end of seven months, the diabetic rats showed evidence for glomerular basement membrane thickening, nodular glomerulosclerosis, mesangial expansion and tubular changes such as proteinuria and glycosuria. This confirmed that the animal model established showed all the prominent histological changes that are observed in human diabetic nephropathy.

Conclusion

From the results, we conclude that there is no need of insulin supplementation for inducing DNP, when the rats are induced with an optimal dose of 45 mg/kg body weight of STZ. Further, the studies that are focused on the therapeutic targets for DNP should maintain the diabetic animals for a minimum period of three months for the animals to attain the pathological changes pertaining to the early stages of DNP.

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