Effect of long acting insulin supplementation on diabetic nephropathy in Wistar rats

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This study was designed to check whether insulin supplementation is crucial for inducing diabetic nephropathy (DNP) in Wistar rats. Diabetes was induced by a single intraperitoneal injection of streptozotocin. The urinary biochemical parameters such as albumin, creatinine and urea nitrogen were monitored every two weeks. The histological changes in the kidney were observed at the end of both fifth and seventh month. Immunohistochemical analyses of VEGF, ERK-1 and NF-κB expression were performed to demonstrate mesangial expansion and glomerulosclerosis, which are the defining histological features of nephropathy. A significant change in the urinary biochemistry was observed in diabetic animals at the end of four months, but the aforementioned quantitative changes were delayed in diabetic animals treated with insulin. At the end of seven months, the diabetic animals showed prominent histological changes such as glomerular basement membrane thickening, nodular glomerulosclerosis and mesangial expansion. However, these changes were not observed in diabetic animals treated with insulin even at the end of the study. From the results, it can be concluded that there is no need of insulin supplementation for inducing DNP, when the animals are induced with an optimal dose of 45 mg/kg body weight of streptozocin.

Keywords: Diabetic nephropathy, Glargine, Long acting insulin, Rat model, Streptozocin

Diabetic nephropathy (DNP) is a chronic kidney disease caused by diabetes mellitus that leads to end stage renal diseases1. Though all patients with diabetes are not prone to DNP, both type-1 and type-2 diabetic patients have an equal chance for getting the disease2. A long standing question pertaining to the diabetic kidney disease is the pathophysiological mechanism involved in the progression of DNP, which is still unanswered3. The mortality of diabetic patients with nephropathy is 30 times higher than the patients without nephropathy4. Therefore, it is the need of the hour to prevent the occurrence or inhibit the progression of DNP in diabetic patients. However, in order to prevent the progression of DNP, the pathophysiology of the disease has to be clearly understood5. In general, the pathophysiology of a disease is well elucidated by clinical observation. But, the clinical studies in DNP are inadequate because the renal disease in diabetic patients develops several years before the clinical manifestations become evident4. Therefore the investigations are solely dependent on the animal models for observing the changes that lead to the progression of DNP6.

Various animal models have been developed till date to elucidate the pathophysiology and to investigate innovative therapies for DNP7-9. The animals were either induced with type-1 or type-2 diabetes and they were allowed to progress to DNP. The rat models generally used for type-2 diabetes are Goto-Kakizaki rats and obese ZUCKER rats10. These rats develop diabetes spontaneously at the age of 14 weeks and if they were maintained in hyperglycemic state for 9-12 months, they progressed to DNP11,12. However, these rats failed to develop nodular glomerulosclerosis and tubulointerstitial fibrosis, which are the most prominent pathological changes of DNP, observed in humans13.

Tesch et al.14 have found that steptozocin (STZ) at a dose of 55, 60, and 45 mg/kg body weight has to be injected in SD, Wistar, and SHR rats respectively for inducing type-1 diabetes that could eventually develop into DNP. Several investigators have used 60 mg/kg body weight of STZ for inducing DNP in
Wistar rats to analyze the effect of various drugs on DNP. Nevertheless, high dose of STZ is found to have non-specific toxicity on the tubular cells of kidney. Therefore the nephropathy developed at high dose of STZ is considered to be a superimposed result of hyperglycemia and STZ induced nephrotoxicity. Simultaneously, a few studies have shown that even a low dose of STZ (35 mg/kg body weight) can induce albuminuria in Wistar rats, reducing the renal toxicity of STZ. Therefore 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 ketonuria and 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 in Wistar rats.

Materials and Methods

Chemicals and reagents—One Touch-Ultra Glucometer (LifeScan, USA), and long acting insulin glargine injection were obtained (Lantus Solostar; Sanofi-Aventis, Germany). Streptozotocin was purchased from Sigma-Aldrich, India. The kits for albumin, glucose, and urea nitrogen quantification were procured from Span Diagnostics Ltd., India. All the other reagents which are not specifically mentioned were purchased from Sisco Research Laboratories (SRL) Ltd., India.

Maintenance of animals—Eight weeks old male albino Wistar rats weighing 200-250 g were used. They were maintained under standard laboratory conditions and supplied with regular pellets and water ad libitum in VIT Animal house, Vellore. The animals were cared as per the principles of the ‘Guide for the care and use of experimental animals’ and the Institutional Animal Ethical Committee approved this entire study (Approval number: VIT/IAEC/II/05/2010).

Study 1: Optimization of STZ dosage—Animals (30) used for this study were segregated into 3 groups of 10 animals each. The animals in all the three groups were administered intraperitoneally with streptozotocin dissolved in freshly prepared 0.1M citrate buffer (pH of 4.5), but at three different doses. Group 1: 35 mg/kg body weight; Group 2: 45 mg/kg body weight; Group 3: 60 mg/kg body weight. The animals were fasted for 16 h before the STZ injection, and after the injection 5% sucrose was supplemented for 48 h in order to prevent from fatal hypoglycemia. The animals were observed as such for 3 months with regular pellets and water ad libitum and the mortality rate in each group was calculated. At the end of each month post-STZ injection, the blood glucose level was measured in the tail vein blood using glucometer. The animals with a random blood glucose level of more than 350 mg/dL were considered diabetic and the percentage of diabetic animals in each group were calculated.

Study 2: Effect of insulin treatment on STZ injected animals

Experimental design: Animals (36) used for this study were allocated into following 3 groups of 12 animals each: Vehicle control (Con), diabetic rats (STZ) and diabetic rats treated with insulin (STZ + Ins). After two weeks of acclimatization, diabetes was induced by single ip injection of STZ dissolved in freshly prepared 0.1M citrate buffer (pH 4.5). The control rats were injected, ip with equal volume of vehicle (0.1M citrate buffer, pH 4.5). One week after STZ injection, blood drawn from the tail vein was analysed for the blood glucose level using glucometer. The animals with a random blood glucose level of more than 350 mg/dL were considered diabetic and were included in the study. In STZ + Ins group, the diabetic rats were administered with long acting insulin at a dose of 2 U/day sc. Every two weeks, two animals from each group were placed in metabolic cages for urine collection. The animals were kept under fasting when they were placed in metabolic cages. The urine collected was filtered and was centrifuged at 2500 rpm for 10 min to remove the contaminants. The urine biochemistry was analysed on the same day of urine collection.

Biochemical analyses—The biochemistry of the collected urine was analysed by standard procedures. Albumin content was quantified by Bromocresol Green method and the urea nitrogen content was measured by the Diacetyl Monoxime method using commercially available kits. Creatinine content was measured by Jaffe method followed by Farrell and Bailey.

Histopathological examinations—Four animals from each group were euthanized at the end of fifth and seventh month by cervical decapitation under...
mild anaesthesia to avoid pain and distress. Kidneys were removed carefully without any damage, washed with phosphate buffer saline (PBS), weighed and fixed in 10% neutral buffered formalin. The kidneys were then processed and embedded in paraffin. Sections (4 μm thick) were cut on a Leica RM 2126 microtome and stained with Haematoxylin & Eosin (H&E), Periodic acid schiff (PAS) and Masson trichrome stains for histopathological analyses. The sections were then photographed under a photomicroscope (Olympus BX51; Olympus optical, Tokyo, Japan) at a magnification of X400. The sections stained with H&E were used to evaluate glomerulosclerosis and the mesangial matrix expansion determined in terms of PAS positive signals present in the mesangial region excluding cellular elements.

Immunohistochemical analysis—Paraffin sections (4 μm thick) were cut, mounted on silanised slides, dewaxed in xylene and rehydrated in graded alcohol. Microwave antigen retrieval was performed in citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ for 15 min. Sections were then washed with water and blocked with normal goat serum for 30 min at room temperature. After washing with phosphate buffered saline containing 0.1% Tween 20, the slides were incubated overnight at 4 ºC with primary antibodies specific for VEGF, ERK-1 and NF-κB. Sections were then processed and embedded in paraffin. The kidneys fixed in 10% neutral buffered formalin. The kidneys were removed carefully without any damage, washed with phosphate buffer saline (PBS), weighed and fixed in 10% neutral buffered formalin. The kidneys were then processed and embedded in paraffin. Sections (4 μm thick) were cut on a Leica RM 2126 microtome and stained with Haematoxylin & Eosin (H&E), Periodic acid schiff (PAS) and Masson trichrome stains for histopathological analyses. The sections were then photographed under a photomicroscope (Olympus BX51; Olympus optical, Tokyo, Japan) at a magnification of X400. The sections stained with H&E were used to evaluate glomerulosclerosis and the mesangial matrix expansion determined in terms of PAS positive signals present in the mesangial region excluding cellular elements.

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Statistical analysis—The data were analysed on Graph Pad Prism 5.01 software and expressed as means ± S.D (n=6 for the fourth month and n=4 for the seventh month). Statistical analysis was performed by Two-way ANOVA followed by Bonferroni post-test to compare the three groups with respect to every month. The results were considered statistically significant, if P < 0.05.

Results

Optimization of STZ dosage—In this preliminary study, the mortality was very high (50%) when the animals were induced with 60 mg/kg body weight of STZ. The increase in mortality has made various investigators to restrict their study period to 1-3 months. Simultaneously, when the animals are induced with a low dose of STZ (i.e.) 35 mg/kg body weight, the percentage of animals having diabetes is only 33.3 though there is no mortality. However, in the present study more than 90% of the animals that were induced with 45 mg/kg body weight of STZ were maintained in hyperglycemic state with only 8.4% mortality at the end of three months. 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.

Effects of insulin injection on polyuria and polydypsia—The most common symptoms of animals induced with diabetes are polyuria and polydypsia. Significant increase in urine volume was observed in animals that were induced with diabetes (P< 0.001) and those that were treated with insulin injection (P< 0.01) compared to that of normal (Table 1). However, there was significant difference between these two groups till the end of four months.

Effect of insulin injection on the biomarker of DNP—Albuminuria is the most common biomarker used for diagnosing DNP. In the present study, a significant increase in the urine albumin level (P< 0.001) was noted in animals injected with STZ.

Table 1—Effect of Insulin on urine biochemistry
[Values mean ± SD of the samples (n=6 for the fourth month and n=4 for the seventh month).]

<table>
<thead>
<tr>
<th>Urinary parameters</th>
<th>Month 4</th>
<th>Month 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con</td>
<td>STZ</td>
</tr>
<tr>
<td>Volume (mL/12 h)</td>
<td>3.2±1.4</td>
<td>24.9±2.2*</td>
</tr>
<tr>
<td>Albumin (mg/24 h)</td>
<td>5.4±1.3</td>
<td>38.9±2.2*</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.4±0.07</td>
<td>0.34±0.01*</td>
</tr>
<tr>
<td>Urea nitrogen (mg/dL)</td>
<td>9.6±0.6</td>
<td>2.7±0.3*</td>
</tr>
</tbody>
</table>

P values: **<0.001; *<0.01; *<0.05 (a,b,c: Con vs STZ groups and Con vs STZ + Ins groups; ***: STZ vs STZ + Ins groups)
(Table 1). However, in the animals that were treated with insulin injection, significant increase in the urine albumin level ($P<0.01$) was observed only from the 5th month. Simultaneously, no significant difference was observed in the urine albumin level beyond fifth month in both the STZ and STZ + Ins groups which showed that insulin can delay but could not prevent the progression of DNP.

**Effect of insulin treatment on kidney damage**—Any kidney damage is reflected by a significant decrease in the level of creatinine and urea nitrogen content in urine and DNP is no exception. In the present study, a significant decrease ($P<0.001$) in creatinine and urea nitrogen content in urine was observed which confirms that the animals in both the STZ and STZ + Ins groups progressed to DNP condition. Further, there exists a significant difference ($P<0.01$) in the urine urea nitrogen and creatinine content between both the groups till fifth month, which means that the insulin injection has delayed the progression of DNP (Table 1).

**Pathological changes in STZ induced diabetic animals in the progression of DNP**—On histopathologic examination of kidney, the diabetic animals showed features of uniform glomerular basement membrane thickening, mesangial expansion and proliferation in more than 25% of the glomeruli at the end of five months. However, there was no evidence of mesangial nodule formation. Tubules showed evidence of protein re-absorption droplets 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 seven months, the diabetic animals revealed evidence for uniform glomerular basement membrane thickening and nodular glomerulosclerosis in the mesangium. Mesangial nodules were found to be PAS positive and the Masson trichrome stain confirmed that the mesangial nodules were not comprised of collagen (Fig. 1). Tubules showed evidence of proteinuria and glycosuria in more than 50% of the glomeruli. Blood vessels revealed evidence of hyaline arteriolosclerosis. However, interstitial fibrosis was not evident even at the end of the study. In diabetic animals that were treated with insulin, though there was evidence of mild mesangial expansion, well formed PAS positive mesangial nodules were conspicuously absent with no change in glomerular basement membrane. The tubules revealed evidence of glycosuria and protein re-absorption droplets in 25% of the cortical surface examined. But, the change in blood vessels was inconspicuous.

**Alteration of protein expressions that mediate the progression of DNP**—ERK-1 and NF-κB were expressed in the cytoplasm and nucleus of occasional tubules (<5% of the cortical surface) in the normal control group. In diabetic animals, there was a marked increase in the expression in terms of intensity and distribution (>50%) of both these markers in the proximal convoluted tubules. In addition, glomerular mesangium also revealed intense expression for ERK-1 while NF-κB was not expressed in the glomerulus. Renal medulla did not show any evidence for the expression of both the proteins. In diabetic animals treated with insulin, the intensity and distribution (25%) of ERK-1 and NF-κB were reduced (Fig. 2). Increased expression (>50%) of VEGF was evinced in the proximal convoluted tubule of diabetic animals. In diabetic animals treated with insulin, the expression was very focal in distribution (<5%) with marked reduction in intensity.

**Discussion**
Though various pathophysiological mechanisms have been proposed on the progression of DNP, hyperglycemia and dyslipidemia are considered to play the lead role in setting the stage for kidney damage in diabetic patients. So, several investigations were focused on maintaining the hyperglycemic state in animal models for inducing diabetic nephropathy. STZ, which is well known for inducing diabetes, has been used at different dosage for maintaining the hyperglycemic state, thus inducing nephropathy in diabetic animals. But, the problem with STZ is twofold. Low dose of STZ was not sufficient to induce nephropathy and at higher dosage, it induced nephrotoxicity rather than nephropathy. Because of these limitations, insulin was chosen as an alternative for maintaining the hyperglycemic state, as followed by several investigators. But it could not be utilized in all situations. When the effect of a drug on nephropathy is being studied, insulin might hamper the results. Since insulin and the drug of interest are supplemented on a daily basis, the mechanistic biological activity of the drug cannot be elucidated. The chance for insulin to interact with the drug and with the metabolism is high, thus confounding the outcome. This made us to investigate whether insulin supplementation is essential in promoting diabetic nephropathy is Wistar rats.
Fig. 1—Figure showing the kidney histological images of control (Con), diabetic (STZ) and diabetic rats treated with insulin (STZ + Ins) at the end of seven months using H&E, PAS and Masson trichrome staining (X400).

Fig. 2—Figure showing the immunohistochemical expression of VEGF, ERK-1 and NF-κB in control (Con), diabetic (STZ), diabetic rats treated with insulin (STZ + Ins) at the end of seven months (X400).
In the present study, the changes in urinary biochemical parameters such as albumin, creatinine and urea nitrogen that were observed at the end of fourth month in diabetic animals were observed only at the end of seven months in diabetic animals treated with insulin. As speculated, insulin delayed the progression of nephropathy, which was substantiated further by the histological and immunohistochemical observations. The diabetic animals showed evidence for glomerular basement membrane thickening, nodular glomerulosclerosis, mesangial expansion, proteinuria and glycosuria at the end of seven months, which are considered to be the prominent histological changes observed in human diabetic nephropathy condition. However, in diabetic animals treated with insulin, there was mild mesangial expansion with tubular changes such as proteinuria and glycosuria. In spite of the hyperglycemic state, which was maintained by insulin supplementation, it was not able to bring forth the pathological changes that are expected in animal models with nephropathy even at the end of seven months.

Hyperglycemia has been proven to induce advanced glycation end (AGE) product formation in diabetic patients. AGE modified proteins interact with the inflammatory cells by interacting with the cell surface molecules such as macrophage scavenger receptor type-2, RAGE and CD36. This ligand-receptor interaction induces post-receptor signaling including the activation of p21ras, ERK-1, ERK-2 and p38 mitogen activated protein kinase. This activates NF-κB, which in turn stimulates the expression of adhesins, chemokines, cytokines and growth factors such as VEGF and TGF-β. VEGF, a potent inducer of vasopermeability, participates in glomerular capillary hyperpermeability of macromolecules, causing diabetic albuminuria. In addition, VEGF also has the ability by itself to stimulate collagen and fibronectin expression, aggravating mesangial matrix expansion and proteinuria. The up-regulation of VEGF and TGF-β result in ERK-1 activation, mediating tubular epithelial cell apoptosis. Further, activation of ERK-1 can also enhance cell proliferation and extracellular matrix protein synthesis. Treatments inhibiting VEGF and ERK-1 have been shown to improve renal function in diabetic patients. In the present study, more than 50% increase in the expression of both VEGF and ERK-1 was observed in diabetic animals, which confirmed that they have encountered chronic nephropathy. However, only a 25% increase in the expression of ERK-1 and NF-κB was observed in diabetic animals treated with insulin compared to control rats. The results were in accordance with the urinary biochemical changes such as albumin, creatinine and urea nitrogen.

NF-κB plays a pivotal role in the pathogenesis of diabetic nephropathy, by regulating the expression of several cytokines and matrix proteins involved in inflammation and immunological responses. Previous studies have documented an increased expression of NF-κB in the tubules and glomeruli of animal models induced with nephropathy. However, in the present study, though there was an increase in the expression of NF-κB in the tubules, the increase was insignificant in the glomeruli. The present results are in accordance with the findings of Mezzano et al., that the activation of NF-κB is focused mainly in the tubular epithelial cells of patients with diabetic nephropathy. This suggests that NF-κB may not play a direct role in mesangial injury in STZ injected diabetic animals.

From the present results, it can be concluded that there is no need of insulin supplementation for inducing DNP, when the animals are induced with an optimal dose of 45 mg/kg body weight of STZ. Further, the studies that are focused on the treatments for DNP should maintain the animals in hyperglycemic state for a minimum period of five months for the animals to attain the pathological changes pertaining to DNP.

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