Chapter 2

Standardization of rat model for diabetic nephropathy
2.1 BACKGROUND

Several animal models have been developed to elucidate the pathophysiology of DNP and to identify innovative therapies for preventing the progression of nephropathy (Marliss et al., 1983; Schmitz et al., 1992; Velasquez et al., 1995). 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 (Janssen et al., 1999). 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 (Popov et al., 2003; Wolf, 2003). However, nodular glomerulosclerosis and tubulointerstitial fibrosis were not observed in these rats, which are the most prominent pathological features observed in human DNP (Riley et al., 1999).

Tesch et al. 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). 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). 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). Simultaneously, in few studies, albuminuria has been induced in Wistar rats even at a low dose (35 mg/kg body weight) of STZ (Kelly et al., 1998; Li et al., 2011). 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 (Davis et al., 2003; Tesch and Allen, 2007). This intended us to investigate if insulin supplementation is crucial for inducing DNP in Wistar rats.
2.2 MATERIALS AND METHODS

2.2.1 CHEMICALS AND REAGENTS

One Touch-Ultra Glucometer (LifeScan, USA) and long acting insulin glargine injection were procured (Lantus Solostar; Sanofi-Aventis, Germany). Streptozotocin was procured from Sigma-Aldrich, India. The kits for albumin, glucose, and urea nitrogen quantification were purchased from Span diagnostics Ltd., India. All the other reagents which are not specifically mentioned were obtained from Sisco Research Laboratories (SRL) Ltd., India. Primary antibodies against VEGF (anti-VEGF, sc-152), ERK (anti-ERK, sc-7383) and NF-κB (anti-NFκB p65, sc-7151) were acquired from Santa Cruz Biotechnolgy, Inc., USA.

2.2.2 MAINTENANCE OF ANIMALS

Eight weeks old male albino Wistar rats weighing 200-250 g were used for the study. The rats were maintained in standard laboratory conditions and supplied with pellets and water ad libitum in VIT University 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 the entire study (Approval number: VIT/IAEC/II/05/2010).

2.2.3 STUDY 1: OPTIMIZATION OF STZ DOSAGE

Thirty rats were utilized for this study and were allocated into three groups with ten rats each. The rats in all the three groups were administered i.p with STZ injection, 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 rats were kept under fasting for 16 hours before the STZ injection, and 5% sucrose was supplemented for 48 hours after the STZ injection in order to prevent the animals from fatal hypoglycemia (Tesch and Allen, 2007). The rats were observed as such for a period of three months and the mortality rate in each group was calculated. At the end of each month post-STZ injection, the blood glucose level was analyzed using glucometer in the tail vein blood. The rats with a
random blood glucose level of more than 350 mg/dl were considered diabetic and the percentage of diabetic rats in each group were calculated.

2.2.4 STUDY 2: EFFECT OF INSULIN TREATMENT ON STZ INJECTED ANIMALS

2.2.4.1 EXPERIMENTAL DESIGN

Thirty six rats were used for this study and were segregated into three groups with twelve rats each. The groups involved were control rats (Con), diabetic rats that were injected with 45 mg/kg body weight of STZ (STZ), diabetic rats that were supplemented daily with 2 units of long acting insulin (STZ + Ins). At the end of every two weeks, two rats from each group were placed in metabolic cages for urine collection and the urine biochemistry was analyzed on the same day of urine collection.

2.2.5 BIOCHEMICAL ANALYSES

The biochemistry of the collected urine was observed using standard procedures. Albumin content was measured by Bromocresol Green method (Gendler, 1984) and the urea nitrogen content was quantified by the Diacetyl Monoxime method (Crocker, 1967) using the commercially available kits. Creatinine content was analyzed based on Jaffe method followed by Farrell and Bailey (1991).

2.2.5.1 MEASUREMENT OF URINARY ALBUMIN

Principle

Determination of albumin in serum or urine is based on the binding ability of albumin with the dye 3,3’,5,5’-tetrabromo-m-cresol-sulfonephthalein (BCG) in acidic medium (pH 4.2). The binding of albumin with BCG is formed as a blue green colored complex, which could be read at 600 nm (Doumas et al., 1971).

Reagents

| Reagent 1: | BCG reagent |
| Reagent 2: | Albumin standard (4 g/dl) |
Procedure

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>BLANK</th>
<th>STANDARD</th>
<th>TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent (R1)</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Standard (R2)</td>
<td>-</td>
<td>10 µl</td>
<td>-</td>
</tr>
<tr>
<td>Test sample</td>
<td>-</td>
<td>-</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

- After the addition of sample, it was mixed well and incubated at room temperature for 1 min
- Immediately, the absorbance of both sample and standard were measured at 600 nm

Calculation

$$\text{Albumin concentration (gm/dl)} = \frac{\text{OD of sample}}{\text{OD of standard}} \times 4$$

2.2.5.2 MEASUREMENT OF URINARY CREATININE

Principle

Creatinine present in the biological sample reacts with alkaline picrate, resulting in the formation of an orange colored complex, the intensity of which is measured at 505 nm. Protein interference from the sample is eliminated using sodium lauryl sulphate. A second absorbance reading after acidifying with 30% acetic acid corrects for non-specific chromogen in the sample (Henry et al., 1974).

Reagents

1) Reagent 1: Creatinine buffer reagent
2) Reagent 2: Creatinine picrate reagent
3) Reagent 3: Creatinine standard (2 mg/dl)
4) Non-hemolyzed serum
5) Urine diluted (1:100) in saline
Procedure

<table>
<thead>
<tr>
<th></th>
<th>STANDARD</th>
<th>TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>50 µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

- Working reagent was prepared by combining equal volumes of reagent 1 and reagent 2
- After the addition of sample, the initial absorbance (A1) at 30 seconds and final absorbance after 120 seconds (A2) were measured for both the sample and standard

Calculation

$$\Delta A = A2 - A1$$

Creatinine concentration (mg/dl) = $$(\Delta AT / \Delta AS) \times 2$$

2.2.5.3 MEASUREMENT OF URINARY UREA NITROGEN

Principle

In alkaline medium, urea is broken down into ammonia and carbon dioxide because of the action of urease enzyme. Ammonia liberated reacts with hypochlorite and salicylate to form dicarboxy-indophenol, which gives the color. The intensity of the color produced is directly proportional to the concentration of the urea present in the sample (Fawcett et al., 1960).

Reagents

1) Reagent 1: Enzyme reagent
2) Reagent 2: Chromogen reagent
3) Reagent 3: Urea standard (40 mg/dl)
4) Non-hemolyzed serum
5) Urine diluted (1:100) in saline

6) Working reagent: Add 10 ml of distilled water to the vial containing the enzyme

**Procedure**

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>BLANK</th>
<th>STANDARD</th>
<th>TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10 µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Mix well and incubate at 37°C for 5 min.

| Reagent 2 | 1 ml | 1 ml | 1 ml |

- It was then mixed and incubated at 37°C for 5 min
- After incubation, the absorbance of both the standard and sample were measured at 600 nm (580-630 nm)

**Calculation**

Urea concentration (mg/dl) = (OD of sample / OD of standard) x 40

2.2.6 HISTOPATHOLOGICAL EXAMINATIONS

Four rats from each group were sacrificed at the end of 20 and 28 weeks under mild anesthesia. Kidneys were removed carefully without any damage, washed with phosphate buffer saline (PBS), weighed and fixed in 10% neutral buffered formalin. The tissues were dehydrated sequentially with increasing concentrations of ethanol (70%, 80%, 90% and 100%) for 1 hour each. The tissues were treated with 100% xylene for 30 min to remove the ethanol. Paraffin wax was added to the sample and was embedded by incubating at 58°C for 2 hours. The paraffin blocks were brought back to room temperature before sectioning. Four micron sections were cut using the microtome and were mounted on glass slides.
Before staining, the slides were deparaffinised by incubating in 100% xylene twice for 3 min each. The slides were washed with decreasing concentration of ethanol (100%, 95%, 80% and 70%) for 3 min each. The slides were rehydrated by immersing the slides in distilled H$_2$O for 5 min. The slides were stained with Hematoxylin & Eosin (H&E), Periodic Acid Schiff (PAS) and Masson Trichrome stains for histopathological analyses. The sections were then studied for the evidence of pathological changes that are observed in human diabetic nephropathy.

### 2.2.7 IMMUNOHISTOCHEMICAL ANALYSIS

Paraffin sections (4 $\mu$m thick) were cut, mounted on silanized slides, dewaxed in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked by incubating in 3% H$_2$O$_2$ for 15 min. After washing with phosphate buffered saline containing 0.1% Tween 20, the slides were incubated overnight at 4°C with primary antibodies specific for VEGF, ERK and NF-Kappa B at 1:200 dilution. The immunoreactivity was performed by incubating at room temperature for 30 min with horseradish peroxidase conjugated goat-anti-rabbit IgG antibody. 3,3′-diaminobenzidine (Dako) was used as the chromogen for detecting the protein expression. Slides were counterstained with hematoxylin, rinsed in tap water, dehydrated, placed in xylene, and mounted. The percentage distribution and the intensity of expression of protein expression were analyzed by a semi-quantitative analysis based on the description by Indian Society of Nephrology. 80-100 glomeruli in the renal cortex were observed for each sample. The number of glomeruli and tubules with positive protein expression was counted and the percentage distribution was calculated. This was examined by two different pathologists who were blinded about the samples.

### 2.2.8 STATISTICAL ANALYSIS

The data were analyzed on Graph Pad Prism 5.01 software and expressed as means ± S.D (n=4). 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 $p < 0.05$. 
2.3 RESULTS

<table>
<thead>
<tr>
<th>After 3 months</th>
<th>Dose of STZ (mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td>% of rats with blood glucose (&gt; 350 mg/dl)</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td>90.9</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2.1: Optimization of STZ dosage

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 (Ohtake et al., 2007; Tuncdemir and Ozturk, 2011). 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>
<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&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Albumin (mg/24 h)</td>
<td>5.4 ± 1.3</td>
<td>38.9 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.4 ± 0.07</td>
<td>0.3 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urea nitrogen (mg/dl)</td>
<td>9.6 ± 0.6</td>
<td>2.7 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 2.2: Effect of insulin supplementation on urine biochemistry**

Values represent the mean ± standard deviation of the samples (n=4). Significant difference between Con Vs STZ groups and Con Vs STZ + Ins groups: <sup>c</sup> p< 0.05; <sup>b</sup> p< 0.01; <sup>a</sup> p< 0.001. Significant difference between STZ and STZ + Ins: <sup>*</sup> p< 0.05; ** p< 0.01; *** p< 0.001, n- not significant.

**2.3.1 EFFECTS OF INSULIN INJECTION ON POLYURIA**

The most common symptoms of diabetes are polyuria and polydipsia. Significant increase in urine volume was observed in both diabetic rats (p< 0.001) and diabetic rats that were supplemented with insulin (p< 0.01) compared to that of control (Table 2.2). However, there was significant difference between these two groups till the end of four months.
2.3.2 EFFECT OF INSULIN INJECTION ON THE BIOMARKER OF DNP

Albuminuria is the most common biomarker used for diagnosing DNP. In this study, significant increase in the urine albumin level (p< 0.001) in rats injected with STZ (Table 2.2) was observed. However, in the diabetic rats that were treated with insulin injection, significant increase in the urine albumin level (p< 0.01) was observed only from the fifth 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 supplementation could delay the progression of DNP.

2.3.3 EFFECT OF INSULIN TREATMENT ON KIDNEY DAMAGE

The kidney damage is revealed by a significant decrease in the level of creatinine and urea nitrogen content in urine and DNP is no exception. In this study, significant decrease (p< 0.001) in both creatinine and urea nitrogen content was observed in urine which confirmed that the rats in both the STZ and STZ + Ins groups have progressed to DNP. Further, there was significant difference (p< 0.01) in the urine urea nitrogen and creatinine content between both the groups till the end of fifth month, which indicated that the insulin supplementation had delayed the progression of DNP (Table 2.2).

2.3.4 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 (Figure 2.1). 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.

Figure 2.1: Kidney histological observation

Figure showing the kidney histological images of control (Con), diabetic (STZ) and diabetic rats treated with insulin (STZ + Ins) at the end of 28 weeks using H&E, PAS and Masson Trichrome staining (Magnification x400).
Figure 2.2: 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).
2.3.5 ALTERATION OF PROTEIN EXPRESSIONS THAT MEDIATE THE PROGRESSION OF DNP

ERK and NF-κB were expressed in the cytoplasm and nucleus of occasional tubules (< 5% of the cortical surface) in the control group. In diabetic rats, there was a marked increase in the expression (> 50%) of both these markers in the proximal convoluted tubules in terms of intensity and distribution (Figure 2.2). Further, intense expression for ERK was observed in the glomerular mesangium while NF-κB was not expressed in the glomerulus. The expression of both ERK and NF-κB was observed mainly in the cortical region with no expression in the medullary region. In diabetic rats treated with insulin, though there was an increase in the expression of both the proteins, the increase was not that significant (< 25%). In diabetic rats, significant increase in the expression (> 50%) of VEGF was observed in the proximal convoluted tubule. However, in diabetic rats treated with insulin, the expression was very focal in distribution (< 5%) with marked reduction in intensity.
2.4 DISCUSSION

Though several pathophysiological mechanisms have been predicted for DNP, hyperglycemia is considered to play the lead role in setting the stage for kidney damage in diabetic patients (Rosario and Prabhakar, 2006). So, it was predicted that for inducing DNP in animal models, the animals have to be maintained in the hyperglycemic condition. STZ, which is well known for inducing diabetes, has been used at different doses for inducing DNP in animal models (Tesch and Allen, 2007). But, there are few drawbacks in using STZ for inducing DNP. Low dose of STZ was not sufficient to induce nephropathy and at higher dosage, the animals encountered nephrotoxicity rather than nephropathy (Kraynak et al., 1995; Kelly et al., 1998). Since it was found that nephropathy could be induced only at high dose of STZ, it was predicted that the mortality in diabetic animals could be reduced by supplementing insulin injections (Tesch and Allen, 2007; Davis et al., 2003). But insulin supplementation could not be utilized in all situations. When the effect of a drug on nephropathy has to be studied, insulin supplementation might hamper the results. Further, the chance for insulin to interact with the drug and with the metabolism is higher, thus confounding the outcome. Since insulin and the drug of interest are supplemented on a daily basis, the mechanistic biological activity of the drug cannot be elucidated. This made us to investigate whether insulin supplementation is essential for inducing nephropathy in diabetic rats.

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 (Table 2.2). 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 (Figure 2.1). This confirmed that the animal model established showed all the prominent histological changes that are observed in human diabetic nephropathy.
However, in diabetic rats supplemented with insulin, though there were severe tubular changes such as proteinuria and glycosuria, the glomerular changes such as mesangial expansion and basement membrane thickening were less prominent. In spite of the hyperglycemic state, which was maintained by insulin supplementation, the rats did not show evidence for all the pathological changes even at the end of seven months.

In hyperglycemic condition, there is evidence for the formation of advanced glycation end product in diabetic patients. AGE products are found to activate several cytokines and growth factors including TGF-β and VEGF in the tubules. The up-regulation of these growth factors enhances ERK activation, thus aggravating the progression of DNP (Cellier et al., 2003). VEGF has the ability to stimulate collagen and fibronectin expression, causing mesangial expansion and proteinuria (Hovind et al., 2000). Since inflammation has been proven to mediate the progression of DNP, treatments inhibiting the upregulation of VEGF and ERK have been shown to improve renal damage in diabetic patients (Chen et al., 2011). In our study, more than 50% increase in the expression of both VEGF and ERK was observed in diabetic rats (Figure 2.2), which evinced that the diabetic rats have encountered chronic nephropathy. However, in diabetic rats supplemented with insulin, only a 25% increase in the expression of ERK and NF-κB was observed (Figure 2.2). The results supported the urinary biochemical changes such as albumin, creatinine and urea nitrogen observed in our study. Since NF-κB regulates the expression of several cytokines and matrix proteins involved in inflammation, it is considered to play a major role in mediating renal injury (Guijarro and Egido, 2001). In animal models of nephropathy, an increased expression of NF-κB has been reported in the tubules and glomeruli (Gomez-Garre et al., 2001). However, in our study, though there was an increased expression of NF-κB in the tubules, the increase was found to be insignificant in the glomeruli. This suggests that NF-κB may not play a direct role in mesangial injury in STZ injected diabetic rats.
2.5 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.

Once the rat model for DNP was established, the query for investigation was that,

What is the level of expression of CD36 in different pathological stages of DNP?