Benificial effects of Bitter guard on neonatal streptozotocin (nSTZ) induced pre-diabetic renal alterations in the SD rat model

K. Siva Kesavarao¹, T. Raghavarao² and P. Suryanarayana².  
¹Lipid Chemistry Division, National Institute of Nutrition, Hyderabad, Telangana, India.  
²Andhra university, Biochemistry Division, Visakhapatnam  
E.mail-kskraok@gmail.com

ABSTRACT

The major purpose of our study is to evaluate the influence of bitter guard on long term pre-diabetic induced renal alterations caused by intraperitoneal induction of neonatal streptozotocin (nSTZ). Two-day old male Sprague Dawley (SD) rat pups (n=30) received a single intra peritoneal injection of STZ (90 mg/kg bw) dissolved in 0.1M citrate buffer, pH 4.5 while Control pups (n=8) received the vehicle alone. All rats were maintained on an AIN-93G/M diet in individual cages and a subset of pre-diabetic animals received 5% bitter guard in the AIN-93 diet. The majority of nSTZ rats exhibited impaired glucose tolerance (IGT) (2h glucose>140mg/dl) or pre-diabetes by 2 months and the same was maintained up to the 10 months as evidenced by OGTT as well as blood glucose levels. Functional abnormalities of the kidney were studied by plasma as well as urinary parameters. Renal pathological changes were observed by H&E staining and immunoblotting was performed to determine the protein expressions of nephrin and podocin. Urinary albumin, urea levels are elevated, whereas plasma albumin levels are decreased in pre-diabetes rats compared to control rats. Protein and mRNA expression levels of nephrin and podocin were lower in PD animals when compared to control. Bitter guard (BG) reduces urinary albumin levels, and also marginally prevented the loss of protein and mRNA expressions of nephrin and podocin. Our findings might provide a basis for long term pre-diabetes induced renal alterations ameliorated by bitter guard, but the exact molecular mechanisms still require further elucidation.  
Keywords: Pre-diabetes, Diabetic nephropathy, bitter guard, STZ

INTRODUCTION

Nephropathy is outlined as functional and structural alterations in the kidney which is also brought on by means of metabolic and cellular abnormalities triggered by elevated blood glucose levels, ultimately leads to renal failure. The transition from the early metabolic abnormalities that precedes diabetes, for example, impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) or pre-diabetes to diabetes could take a very long time. Nevertheless, present estimates point out that the fundamental contributors with these pre-diabetic states eventually improve diabetes mellitus [1-3]. In up to date years, numerous experiences have proven that an impairment of podocytes, which might be the fundamental cell type of the glomerulus, plays a most important function in the pathogenesis of DN [4]. Podocytes, which are positioned at the interior lining of the glomerular capsule, together with the basement membrane and endothelial cells represent the glomerular filtration barrier. Podocyte hypertrophy, degeneration, shedding and loss mainly influence in a disruption of the glomerular filtration barrier, which leads to albinuria and deterioration in nephropathy. The podocyte proteins, in particular nephrin and podocin are the essential elements of the slit diaphragm required to keep the selective permeability of the glomerular filtration barrier [5, 6]. Research related to changes within the expression of nephrin and podocin proteins has turned out to be more popular in recent years because proteinuria, glomerulosclerosis, and renal function deterioration can be triggered by the down regulation of nephrin and podocin, which outcomes in structural and functional damage to the podocyte slit diaphragm [7].

Momordica charantia commonly known as bitter gourd or bitter melon is a native of the Indian subcontinent. It is widely known for its antidiabetic/ hypoglycaemic effect and due to the same it is
popularly termed as vegetable insulin. It is widely used for anti-diabetic treatment and testing of its anti-diabetic principle considering that of an extract bought from the bitter guard fruit has been reported to be homologous to pancreatic insulin and its hypoglycemic action has been well established based on clinical tests on humans as well as in animal models [8-12].

Nephropathy is one of the fundamental microvascular complications also occurs in pre-diabetic conditions [13], however the pathophysiology of this complication is not fully understood. Understanding the pathophysiology of nephropathy in IGT/IFG or pre-diabetic state will aid to undertake suitable interventions at an early stage of disease. For this reason, the motive of this work was once to clarify whether renal alterations are already present in a pre diabetic state, mechanisms concerned in it and to be investigated defending outcomes of bitter guard on nSTZ induced pre-diabetic rat model.

MATERIAL AND METHODS
Preparation of bitter gourd powder:
Fresh bitter gourd was obtained from the nearby market and was lyophilized. The dried bitter gourd was pulverized to powder form and added to AIN-93 diet and was fed to the experimental animals.

Animals and Diets
Male Sprague-Dawley rat pups weighing 8-9 g (obtained from the National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India) had been used in this study. The animals were housed at an ambient temperature, humidity controlled rooms with 12 hours light-dark cycle with free access to water. Body weights of rats were monitored once a month throughout the study.

Induction of pre-diabetes and experimental groups:
Male two days old SD rat pups were selected for this experiment. Pre-diabetes was induced in overnight fasted rat pups by a single intraperitoneal injection (i.p) of freshly prepared STZ (90 mg/kg body weight). STZ was dissolved in citrate buffer (pH 4.5). Control pups received only citrate buffer. Only nSTZ injected rats having postprandial blood glucose levels more than 140 mg/dL and fasting blood glucose levels between 100-125 mg/dL at 2 months after STZ injection were considered as pre-diabetic and included in the study.

Experimental Design
A total of 45 rat pups was used. The rats were divided into three groups after the induction of pre-diabetes with STZ. The experimental period was 11 months. Group I (control rats) received saline which was maintained on AIN 93 diet. Group II was pre-diabetic rats maintained on AIN 93 diet and group III pre-diabetic rats received 5% bitter gourd mixed with AIN 93 diet (PD+BG, n=9).

Fasting and postprandial blood glucose:
Fasting and postprandial blood glucose levels in these experimental animals were monitored every month by glucometer (One Touch Horizon).

Oral glucose tolerance test (OGTT):
OGTT was conducted after 2nd and 10th month of STZ injection on overnight fasted rats by administering glucose orally as a bolus, at a dose of 2.0 g kg⁻¹ of body weight. Blood samples have been collected at 0, 30, 60 and 120 minutes time intervals for estimating plasma insulin glucose and concentrations to assess insulin resistance and impaired glucose tolerance (IGT).

Biochemical parameters.
Plasma glucose used to be measured with the aid of the glucose oxidase–peroxidase (GOD–POD) procedure using a commercially available kit (BioSystems, Spain). HbA1c (RBC), albumin, creatinine and urea (plasma and urine) were estimated utilizing commercially available kits (BioSystems, Spain).

Blood, kidney collection and processing
Blood used to be collected once a month from the retro-orbital plexus for estimation of glucose. 24 h urine was gathered from experimental animals by placing them in metabolic cages. At the finish of 10 months duration of pre-diabetes, the animals have been sacrificed through CO2 asphyxiation. Kidneys have been perfused through the abdominal aorta with 100 ml of normal saline. The left renal vein used to be punctured to allow the perfusate to drain and the kidney used to be removed instantly and placed in four% paraformaldehyde for subsequent histologic reports. The rest of the kidneys were snap frozen in liquid nitrogen and stored at -80°C for isolation of both RNA and proteins for further studies.

Histopathology
For histological studies kidneys were dissected out and fixed in 4% paraformaldehyde followed by paraffin sections and stained with hematoxylin and eosin staining. Kidney morphology was examined under the microscope.
Quantitative real-time PCR
Total RNA extraction was done from the frozen kidney by Trizol method and quantified by Nanodrop spectrophotometer (ND1000) and the integrity of RNA was checked on denaturing MOPS-Formaldehyde gel. 1μg of RNA was reverse transcribed using Transcript or First Strand cDNA Synthesis Kit (Roche). The real time PCR was performed with the use of Applied Biosystems 7500 instrument with SYBR green (Applied Biosystems) on cDNA samples using the primers listed in [Table-1] And reaction stipulations had been as follows: initial RT for 10 min at 25°C, adopted by way of 37°C for 120 min and inactivation of reverse transcriptase at 84°C for 5 min. At the end of the run, specific products generated for each set of primers were examined by melting curve analysis. The relative expression levels of each gene of interest were normalized by subtracting the corresponding beta-actin threshold cycle (CT) values by using the DDCT comparative method. From each group three samples were used and each sample was run in triplicate. The reaction conditions have been as follows: 40 cycles of initial denaturation temperature at 95°C for 30 seconds, followed through annealing at 52°C for 40 seconds and extension at 72°C for 1 min and product specificity was once analyzed by utilizing melt curve analysis.

Western blot analysis
Tissue lysates had been prepared in homogenization buffer containing protease inhibitor (Complete mini, Roche, Mannheim, Germany), 1 mM sodium orthovanadate and 50mM NaF. Lysates had been centrifuged at 12,000g and aliquots of the supernatants were separated by means of 12% SDS–PAGE and transferred to nitrocellulose membrane (PALL, USA). After probing with corresponding primary antibodies antigen–antibody complexes were detected with horseradish peroxidase-labeled secondary antibodies. Immunoreactive bands were visualized using enhanced chemiluminescence reagents (Bio-Rad) and quantified using Image J software.

Statistical analysis
By using SPSS 19.0 software all statistical analyses were performed. All quantitative data were presented as mean ± standard deviation (SD). Differences among means were analyzed by one-way ANOVA test, followed by Tukey HSD test and student test. Statistical significance was set at p<0.05.

RESULTS
Food intake, body weights and blood glucose levels:
All untreated PD and BG treated PD rats showed a marginal increase in fasting blood glucose, but there was a significant (p<0.01) increase in postprandial blood glucose (11.92±3.98 mmol/l) levels of untreated PD rats when compared to control (6.32±0.31 mmol/l). There was a slight decrease in body weights in both PD and BG treated PD rats when compared to control. There was a marginal increase in mean food intake in PD rats when compared to control rats. However, a significant (p<0.01) decrease in food intake was observed in BG treated PD rats when compared to control rats. PD rats. There was a slight decrease in body weights of PD rats and BG treated PD rats when compared to control rats by the end of the experiment [Table-1].

OGTT after 2nd and 10th month:
nSTZ rats developed IGT associated pre-diabetes by two months. In the present study, after 2nd and 10th month of STZ injection, OGGT graph showed higher plasma glucose levels in all the time points except 0 min in nSTZ injected rats when compared to control, where-as insulin levels are lower in PD rats compared to control in all time points except 120 min [Fig-1], indicating the development of IGT or pre-diabetes by two months and maintained pre-diabetes up to 11 months. Moreover, after 10 months of STZ injection, BG treated PD rats showed a slightly lower plasma glucose and insulin levels when compared to PD [Fig-1C and 1D].

HOMA-IR and HbA1C:
There was a decrease in HOMA-IR index in PD rats when compared to control rats, which was restored in BG treated PD rats [Table-2]. Further, HbA1C levels were slightly increased in PD rats when compared to control rats, which were restored to a maximum extent when PD rats fed with BG rats in all the time points except at 0 min fasting plasma glucose [Fig-1B].

Urine and Plasma parameters:
To assess long-term pre-diabetes on the development of renal abnormality, we estimated urinary albumin, creatinine and urea in control, pre-diabetic rats and pre-diabetic rats fed with BG. Pre-diabetic rats excreted elevated levels of albumin compared to control rats, indicating altered renal function due to pre-diabetes. Whereas urinary albumin content was decreased in BG fed pre-diabetic animals (Table-3). Similarly a pre-diabetic rats excreted higher amount of urinary urea when compared to control animals. Feeding of BG to pre-diabetic rats reduced urinary urea levels. These results indicated that BG has prevented renal abnormalities induced due to pre-diabetes. (Table-3). However, there was no difference
in urinary creatinine levels between groups. There was no difference in plasma albumin, creatinine, urea and blood urea nitrogen all groups (Table-4). There was a slight increased glomerular filtration rate per body weight (GFR/b.w) in PD rats (2.89±0.63) when compared to control rats (2.93±0.72). Where as BG treated PD rats shown marginally reduced GFR (2.81±1.29) when compare to PD rats.

**Kidney morphology:**
In the kidney H&E sections of pre-diabetic group rat kidney shown modest glomerular lesions compared to control group rat kidney sections, whereas BG treated PD rat kidney shown only minute glomerular lesions compared to untreated PD rats [Fig-2].

**Alterations of renal protein expression:**
We used western blot methods to study the expressions of key protein molecules such as Nephrin and Podocin in control, PD and BG treated PD rat kidney. As expected, there was a slight decrease in the relative expression of nephrin proteins and there was a significant (P<0.05) decrease in podocin protein in PD rat kidneys when compared to control. BG marginally prevented the loss of nephrin and podocin levels [Fig-3A and 3B].

**Renal gene expression:**
Real time analysis showed down regulated mRNA expressions of nephrin and podocin in PD rats when compared to the control, indicating the renal abnormalities clearly manifested in pre-diabetes, these results further support the immunoblot results. Feeding of BG to PD rats had improved Nephrin and Podocin m-RNA expressions [Fig-3C].

**DISCUSSION**
Streptozotocin is a broadly used inducer of diabetes in experimental animals, it is enhancer of selective destruction of pancreatic islet cells of Langerhans [14]. In the present study, we developed pre-diabetes rat model by intra-peritoneal (IP) injection of STZ to 2 days old SD rat pups lead to hypo insulinemia and hyperglycemia as an evidenced by OGTT insulin and blood glucose levels at 11 months [Fig-1and Table-1]. Pre-diabetic rats showed a significantly increased postprandial glucose levels compared to control rats because STZ selectively destructs β-cells of the islets of Langerhans in the pancreas, resulting in inhibition of insulin synthesis and elevation of blood glucose level due to, first of all, a diminished entry of glucose to peripheral tissues, muscle and adipose tissue, secondly, increased glycogen breakdown and eventually, accelerated gluconeogenesis and hepatic glucose production [15]. BG fed PD rats shown decreased postprandial glucose levels compared to pre-diabetes rats in accordance with previous studies [16, 17].

**Figure 1:** Glucose and insulin response during OGTT at three (A, B) and ten months (C, D).
Fig 2: Morphology of kidney by H&E staining in control (A), pre-diabetic (B) and pre-diabetic rats fed with BG (C).

![Morphology of kidney by H&E staining](image)

Fig 3: Pre-diabetes induced altered protein expressions in the kidney by western blot (A), Desitometric analysis (B) and gene expression by qRT-PCR (C).

![Protein expression and gene expression](image)

Protein expression of nephrin and podocin were studied in control, PD and PD+BG rats kidneys using western blot (Panel A) and concentration of these proteins were measured by densitometric analysis using Image J software (Panel B). Expression of nephrin and podocin genes in control, PD and PD+BG rat kidneys were analyzed by qRT-PCR (Panel C). Group (*, p<0.05)

### Table 1: List of primers and their sequence used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephrin</td>
<td>5'ACA GCG TGC TGG TGA TGA CTG T 3'</td>
</tr>
<tr>
<td></td>
<td>5'TGG TAA TGG CGC TTG GGG GAAA 3'</td>
</tr>
<tr>
<td>Podocin</td>
<td>5'AGC CAT CCA GTT CCT GTT GCA A 3'</td>
</tr>
<tr>
<td></td>
<td>5'TGC CCC AAA CAC AGG TCA CTG A 3'</td>
</tr>
<tr>
<td>Beta-actin</td>
<td>5'CGA CAA CGG CTC CGG CAT GT 3'</td>
</tr>
<tr>
<td></td>
<td>5'GGG GCC ACA CGC AGC TCA TT 3'</td>
</tr>
</tbody>
</table>

### Table 2: General characteristics of control, PD, PD+BG group rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>PD</th>
<th>PD+BG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake (g/day/rat)</td>
<td>19.62±2.83</td>
<td>20.26±3.07</td>
<td>18.66±3.3255</td>
</tr>
<tr>
<td>Body weight (g/rat)</td>
<td>470±29</td>
<td>440±48</td>
<td>433±36</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>4.45±0.57</td>
<td>5.17±0.73</td>
<td>5.00±0.42</td>
</tr>
<tr>
<td>2h postprandial blood glucose (mmol/L)</td>
<td>6.32±0.31</td>
<td>11.92±3.90**</td>
<td>10.47±1.79</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.85±0.14</td>
<td>0.70±0.24</td>
<td>0.80±0.27</td>
</tr>
<tr>
<td>HbA1C</td>
<td>7.23±0.28</td>
<td>6.30±0.80</td>
<td>7.30±0.44</td>
</tr>
</tbody>
</table>

Pre-diabetic (PD); PD+BG: Pre-diabetic + Bitter gourd; Values are mean ±SD, n=9-10 animals; **p<0.01.
Table 3: Urinary parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>PD</th>
<th>PD+BG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (mg/24h)</td>
<td>18.13±3.95</td>
<td>57.81±29.72</td>
<td>42.45±25.87</td>
</tr>
<tr>
<td>Creatinine (mg/24h)</td>
<td>17.84±2.09</td>
<td>15.18±4.96</td>
<td>15.62±6.71</td>
</tr>
<tr>
<td>Urea (mg/24h)</td>
<td>650±28.33</td>
<td>674±55</td>
<td>601±110</td>
</tr>
<tr>
<td>Water intake (ml/24h)</td>
<td>20.20±9.93</td>
<td>23.83±3.12</td>
<td>28.83±9.60</td>
</tr>
<tr>
<td>Urine output (ml/24h)</td>
<td>9.14±0.89</td>
<td>6.58±3.36</td>
<td>13.00±10.58</td>
</tr>
</tbody>
</table>

Table 4: Plasma parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>PD</th>
<th>PD+BG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (mg/dL)</td>
<td>25.06±1.72</td>
<td>23.75±2.17</td>
<td>23.97±1.55</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.911±0.050</td>
<td>0.890±0.055</td>
<td>0.872±0.155</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>29.14±3.25</td>
<td>29.90±4.64</td>
<td>27.53±3.01</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>13.58±1.51</td>
<td>13.93±2.16</td>
<td>12.83±1.40</td>
</tr>
</tbody>
</table>

Our study demonstrates no variations on glycemia in the fasting state, but a significant increase in the postprandial state in PD rats, along with impaired glucose tolerance. The mechanisms underlying the progression of diabetic nephropathy had been widely discussed and seem to involve elaborate and multifactorial influences. Certainly, as in diabetes, hyperglycemia, hyperinsulinemia and hyperlipidemia probably the preliminary triggers of enormous cellular and molecular alterations ultimately important to renal functional and structural impairment, key aspects of the renal dysfunction related to nephropathy.

Podocytes, which might be some of the predominant cell types that represent the glomerular filtration barrier injured in diabetes, leading to albuminuria and nephropathy [18]. Foot processes, which interdigitate to form bridging structures often called slit diaphragms, are characteristic features of podocytes. The primary proteins, together with nephrin and podocin keep the integrity of the podocyte slit diaphragm [19]. Nephrin is the earliest discovered protein that is specifically located in the podocyte slit membrane area. Podocin connects nephrin and CD2AP via its C-terminus to type a “zipper-type” filtration barrier and to maintain the natural constructions and features of the glomerular filtration membrane. Earlier experiences have proven that the expression of nephrin and podocin was lessen in diabetic rats [20], in the present study we also discover low levels of nephrin and podocin in both mRNA and protein expressions in PD rats compared to control. In diabetic patients, reduced expression of nephrin and podocin occurred earlier than ultrastructural changes of the podocyte and the occurrence of proteinuria [21]. Therefore, nephrin can be used to potentially indicate early glomerular podocyte injuries [22]. In the present study, elevated urinary albumin levels in pre-diabetes rats may be reduced nephrin and podocin expressions in both protein as well as mRNA level. Albuminuria and nephropathy correlate with the reduced expression the podocyte membrane-associated proteins, nephrin and podocin. Changes in expression of nephrin and podocin might change the permeability of the glomerular filtration membrane by affecting the integrity of the foot processes leads to the excretion of more urinary albumin in this pre-diabetes rat model.

In summary, this study confirmed that albuminuria and renal functional and structural alterations are an early consequence of nephrin and podocyte reduction in the renal tissue. Bitter guard partially prevented kidney lesions and maintain the proper expression and distribution of nephrin and podocin proteins might be reduced glucose levels.

CONCLUSION
Our findings might provide a basis for long term pre-diabetes induced renal alterations attenuated by bitter gourd, but the exact molecular mechanisms still require further elucidation.

ACKNOWLEDGEMENTS
K.S.K. Rao received a research fellowship from the Indian Council of Medical Research, Government of India.

FUNDING SOURCES
P.S.N. Received grants from the Department of Biotechnology, Government of India (Grant No: BT/PR3446/BRB/10/969/2011) and National Institute of Nutrition (Indian Council of Medical research, Government of India) for intramural funding (#12-BS11).
Conflict of Interest: None declared

REFERENCES


©2019 AELS, INDIA