Nephroprotective Effect of Naringenin against Multiple Low Dose Streptozotocin (MLDSTZ) Induced Renal Damage in Mice

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ABSTRACT

Streptozotocin, a widely used diabetes-inducing agent, is known to cause severe damage to organs that include pancreas, liver and kidneys. This organ damaging effect of streptozotocin is primarily due to its ability to elevate blood glucose, which in turn cause oxidative stress and many metabolic abnormalities. Therefore, antioxidant compounds that can inhibit STZ-induced ROS are potential anti-diabetic agents and help in protecting organs from STZ-mediated damage. Hence, in this study the ability of naringenin, a naturally occurring flavonoid, to protect kidneys from the damage caused by the administration of MLDSTZ in mice was evaluated. Mice treated with intraperitoneal injections of 50mg/kg body weight streptozotocin, for five days, have developed nephropathy, as confirmed by elevated urea, uric acid, creatinine, bilirubin, albumin, and serum thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides. In addition, a study decline in (a) antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT); (b) glutathione (GSH) and glutathione peroxidase (GPx) and glutathione-S-transferase (GST) was observed in the lysates collected from kidneys. Oral administration of naringenin at 50mg/kg and 100mg/kg body weight for 45 days to STZ treated mice mitigated the complications of nephrotoxicity as demonstrated by normal (or close to normal) levels of renal markers and antioxidants. Furthermore, histopathological examination of kidney sections showed recovery of normal morphology of tissues indicating that naringenin protects mice from STZ-induced kidney damage.

Keywords: Naringenin, Streptozotocin, Diabetic nephropathy, Antioxidants.

INTRODUCTION

Streptozotocin (STZ) is one of the widely used naturally occurring diabetes-inducing compounds isolated from Streptomyces achromogenes. Chemically it is known as N-(Methylnitrosocarbamoyl)-±-D-glucosamine1. Prior studies have shown that administration of STZ by oral or intravenous routes induces diabetes in mice and rats by destroying pancreatic beta cells2. In addition to beta cell destruction, STZ is also known to cause damage to other organs such as kidneys (Nephrotoxicity), liver (Hepatotoxicity) and heart (Cardiotoxicity) either through its direct effect or through the complications induced by elevated blood glucose levels3. Despite these toxic effects, streptozotocin is used as a chemotherapeutic agent for treating pancreatic cancers 4. Therefore
strategies to overcome the organ toxicity caused by STZ are urgently warranted. Use of natural products, such as flavonoids, is one such strategy to mitigate the complications of STZ-induced damage. Flavonoids act as antioxidants to inhibit reactive oxygen species, which otherwise are detrimental to kidneys and other organs in the body. Although various flavonoids such as resveratrol and hesperedin are well explored for their ability to reduce the complications associated with STZ administration, their usage is highly discouraged due to poor bioavailability and limited biological activity. Hence, an intense search for more bioavailable and effective natural product(s) continued, which identified naringenin as a possible candidate for further testing.

Naringenin is a bioactive flavonoid (Figure 1), widely distributed in citrus fruits. Prior studies have demonstrated that naringenin could safeguard cells from the damage caused by oxidative stress and inflammatory cytokines. Supporting this a study showed the protection from cisplatin induced kidney damage by naringenin treatment. Similarly, other studies have reported protection from doxorubicin-induced cardio-toxicity and streptozotocin (STZ) induced hepatic inflammation. Moreover, recently, naringenin has been reported to be a better agent compared to hesperedin (flavanones) in protecting mitochondrial and cellular membranes. Hence, in the present study an attempt was made to evaluate the potency of naringenin to protect kidneys from multiple low-dose streptozotocin induced damage in mice.

MATERIALS AND METHODS

Materials
Naringenin, Streptozocin, Glibenclamide, and Carboxymethyl Cellulose were procured from Sigma Chemical Company (St Louis, MO). Other chemicals used for this study are of analytical grade.

Animal experiments
Animal experiments were carried out after receiving approval from the Institutional Animal Ethics Committee (JSSMC/IAEC/18/5675/DEC2013). In brief, male mice weighing about 25-30g were maintained as per the guidelines given by the National Institute of Nutrition, Hyderabad, India and used for the study.

Treatment with streptozocin
Mice were injected with STZ (dissolved in 0.1M citrate buffer, pH 4.5) intra-peritoneally at a dose of 50mg/kg/day for 5 consecutive days and blood (50 to 100µL) collected from the tail vein. Glucose, in the collected blood, was estimated using Accu-Check Active glucometer to determine whether streptozotocin is functionally active and induced diabetes. Mice with blood glucose concentration >250mg/dL were selected for testing the potency of naringenin for mitigating STZ-induced nephrotoxicity as detailed below. First, 24 STZ treated and 12 normal mice were divided into a total of 6 groups as shown in Table 1

Naringenin (NAR) and glibenclamide (GLC) were suspended in 0.5% carboxymethyl cellulose. At the end of experiment (after 24h of last naringenin administration), the animals were deprived of food overnight and the blood was collected by cardiac puncture. Serum was separated, and stored at -20°C until analysis. Kidney tissues, from each animal, were minced and homogenized in 100mM Tris-HCl buffer, pH 7.4, and centrifuged. The supernatant was used for estimation of TBARS, lipid hydroperoxides, SOD, CAT, GSH, GPX and GST.

Estimation of biochemical constituents in the serum
Concentration of biochemical constituents, whose levels in serum go high when there is a damage to kidneys, such as urea, uric acid, creatinine, albumin and bilirubin was estimated using commercially available kits from Coral Clinical System, Goa, India.

Measurement of STZ-induced oxidative stress
The levels of Lipid peroxidation, in terms of the levels of TBARS formed, was measured using Niehaus and Samuelsson, 1968 method and hydroperoxides was measured by the method of Jiang et al., (1992). Further, antioxidant enzymes (a) Superoxide dismutase by Kakkar et al., (1984); (b) Catalase by Sinha et al., (1972); (c) Glutathione peroxidase by Rotruck et al., (1973); and Glutathione "S"-Transferase by Habig et al.,
and antioxidant molecules such as reduced Glutathione by Ellman’s et al., (1959) levels were measured in the renal tissues. For detailed experimental procedures, the readers are suggested to refer the cited references.

**Histological observations of kidney**
At the end of the experiment, kidney tissues were fixed in 10% buffered formalin for 24h, and dehydrated using ethanol and xylene for 30min each. Tissues were then incubated in liquid paraffin (58°C) twice for 60min each. Tissue blocks were sectioned into 5½m thickness and stained using hematoxylin-eosin. The morphological changes were observed under the light microscope.

**Statistical analysis**
All the obtained results were expressed as mean ± SEM of six separate experiments (n = 6). The statistical significance was performed using one-way analysis of variance (ANOVA), SPSS statistics 20 (SPSS, Cary, NC, USA) followed by the Tukey’s post hoc test. P<0.05 was considered as significant.

**RESULTS**

**Oral administration of naringenin reduces the streptozotocin-induced renal damage**
It is well established that damage to kidneys by streptozotocin administration elevates serum urea, uric acid, creatinine, albumin and bilirubin. Therefore, to test whether naringenin administration reduces these elevated serum biochemical constituents, first, mice were treated with STZ and damage to kidneys confirmed (Table 1). Next, these mice were treated with 50mg/kg and 100mg/kg body weight naringenin for 45 days, and changes in the biochemical constituents, listed above, measured and compared (before and after naringenin treatment). STZ treatment significantly (P<0.05) increased serum (a) urea from 24.73±0.9 mg/dL to 56.13±4.1 mg/dL; (b) uric acid from 3.81±0.2 mg/dL to 9.38 ±0.2mg/dL; (c) creatinine from 0.69±0.1mg/dL to 1.67±0.3mg/dL; (d) bilirubin from 0.57±0.3mg/dL to 1.28±0.1mg/ dL; (e) albumin from 3.39±0.2g/dL to 5.69±0.4g/dL (Table 1). However, administration of naringenin (50 and 100mg/kg b.w.) and positive control compound glibenclamide (600µg/kg b.w.) to STZ-treated mice significantly (P<0.05) decreased the levels of these constituents.
biochemicals postulating the protective effect of naringenin against STZ-triggered toxicity. For example, the concentration of urea in serum has come down from 56.13±4.1 mg/dL in STZ treated mice to 42.70±2.5 mg/dL and 31.12±1.4 mg/dL, respectively in 50 & 100mg/kg b.w. naringenin treated animals. Similarly other biochemical constituents also showed significant dose dependent reduction upon treatment with naringenin (Table 1)

**Table 2: Protective effect of naringenin on renal damage induced by streptozotocin in normal and experimental mice**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urea (mg/dL)</th>
<th>Uric acid (mg/dL)</th>
<th>Creatinine (mg/dL)</th>
<th>Bilirubin (mg/dL)</th>
<th>Albumin (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.73±0.9</td>
<td>3.81±0.2</td>
<td>0.69±0.1</td>
<td>0.57±0.3</td>
<td>3.39±0.2</td>
</tr>
<tr>
<td>C+NAR (100mg/kg)</td>
<td>24.75±0.9</td>
<td>3.79±0.3</td>
<td>0.64±0.0</td>
<td>0.61±0.3</td>
<td>3.33±0.1</td>
</tr>
<tr>
<td>STZ Control</td>
<td>56.13±4.1 *</td>
<td>9.38±0.2 *</td>
<td>1.67±0.3 *</td>
<td>1.28±0.1 *</td>
<td>5.69±0.4 *</td>
</tr>
<tr>
<td>STZ + NAR (50mg/kg)</td>
<td>42.70±2.5 4</td>
<td>7.83±0.3 4</td>
<td>1.19±0.1 4</td>
<td>0.82±0.0 4</td>
<td>5.08±0.4 4</td>
</tr>
<tr>
<td>STZ + NAR (100mg/kg)</td>
<td>31.12±1.4 4</td>
<td>5.47±0.2 4</td>
<td>0.84±0.1 4</td>
<td>0.64±0.1 4</td>
<td>4.10±0.2 4</td>
</tr>
<tr>
<td>STZ + GLC (600µg/kg)</td>
<td>29.03±1.4 4</td>
<td>4.46±0.2 4</td>
<td>0.76±0.0 4</td>
<td>0.61±0.1 4</td>
<td>3.66±0.2 4</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE from six mice in each group. Statistical analysis was performed by one-way ANOVA, followed by Turkey post hoc test. *STZ control mice were compared with control mice. **STZ+NAR (50 and 100mg/kg b.w.) and STZ+GLC (600µg/kg b.w.) treated mice were compared with STZ control mice; P < 0.05.
and GST) and other biomolecules (Glutathione). Therefore, the activities of major antioxidant enzymes were measured in control and treated mice. SOD catalyses the dis-mutation of superoxide radicals to H_2O_2 thereby reducing the possibility of superoxide anion interacting with nitric oxide to form reactive peroxynitrite. The SOD activity in the tissues was assayed according to the method of Kakkar et al., (1984). The SOD activities were significantly (p<0.05) decreased in the kidney tissue of STZ control animals (from 11.70±0.1 to 6.2±0.1) (Figure 3A). Oral administration of naringenin (50mg/kg and 100mg/kg body weight) and glibenclamide (600µg/kg b.w.) significantly (p<0.05) augmented the levels to near control levels (Figure 3A). Treatment of control animals (exposed to vehicle 0.5% carboxymethyl cellulose) with 100mg/kg body weight naringenin did not change SOD levels (Figure 3A).

Catalase is a tetrameric hemin-enzyme, which decomposes hydrogen peroxide into H_2O and oxygen. The catalase activity (µmoles of H_2O_2 consumed/min/mg protein) in the tissues was assayed by the method developed by Sinha et al., (1972). The CAT activity significantly (p<0.05) decreased from 42 units in control animals to 28 units in STZ-treated animals (Figure 3B). Oral administration of naringenin (50mg/kg and 100mg/kg body weight) and glibenclamide (600µg/kg b.w.) significantly (p<0.05) augmented the levels to near control concentration (Figure 3B). For example, whereas 100mg/kg body weight naringenin

Increased lipid peroxidation can be due to increased oxidative stress in the cell as a result of impaired activities of antioxidant defense enzymes. The STZ control animals showed a significant (p<0.05) increase in the levels of TBARS and hydroperoxides when compared with the control group (Figure 2A, 2B). Oral administration of naringenin (50 and 100mg/kg b.w.) and glibenclamide (600µg/kg b.w.) showed significantly decreased TBARS and hydroperoxides to near normal levels in kidney tissues of STZ mice (Figure 2A, 2B). Data are expressed as mean ± SE from six mice in each group. Statistical analysis was performed by one-way ANOVA, followed by Turkey post hoc test. *STZ control mice were compared with control mice. **STZ+NAR (50 and 100mg/kg b.w.) and STZ+GLC (600µg/kg b.w.) treated mice were compared with STZ control mice; P < 0.05.
Persistent hyperglycemia leads to increased production of free radicals. STZ control mice showed a significant (p<0.05) decrease in the levels of antioxidants when compared with the control group (Figure 3 A-E). Oral administration of naringenin (50 and 100mg/kg b.w.) and glibenclamide (600µg/kg b.w.) showed significantly increased antioxidants to near normal levels in kidney tissues of STZ mice (Figure 3 A-E). Data are expressed as mean ± SE from six mice in each group. Statistical analysis was performed by one-way ANOVA, followed by Turkey post hoc test. *STZ control mice were compared with control mice. †STZ+NAR (50 and 100mg/kg b.w.) and STZ+GLC (600µg/kg b.w.) treated mice were compared with STZ control mice; P < 0.05.

Fig. 3: Effect of naringenin on the activities of SOD, CAT, GPX, GTS and GSH in the kidney tissues of control and experimental mice
increased the catalase activity to ~40 units, the administration of 600µg/kg body weight glibenclamide elevated the levels to about 42 units (Figure 3B). No significant changes were observed in mice treated with 100mg/kg body weight naringenin in comparison to control animals (exposed to vehicle 0.5% carboxymethyl cellulose) demonstrating that naringenin administration has no major impact on catalase activity (Figure 3B).

Figure 4 represents the H & E staining of kidney tissues of control and experimental mice. Control and C+NAR (100mg/kg b.w.)- Shows renal parenchyma with normal glomeruli (orange arrow) and tubules; STZ control- necrosis, bowman’s space expansion (yellow arrow) and swelling of the epithelial cells; STZ+NAR (50mg/kg b.w.)- Moderate necrosis (orange arrow). STZ+NAR (100mg/kg b.w.)- Absence of fibrosis, slight congestion of glomerular tufts, and normal glomeruli (brown arrow). STZ+GLC (600µg/kg b.w.)- Normal histoarchitecture of the kidney (brown arrow).

GPX, a selenium-containing peroxidase, it decomposes H₂O₂ and lipid peroxides by using GSH, and protects the cells from free radicals. The activity of glutathione peroxidase was determined by the method of Rotruck et al., (1973). The GPX activity (µmoles of GSH oxidized/min/mg protein) was significantly (p<0.05) decreased from ~8.5µmoles in control animals to ~4.0µmoles in STZ-treated animals (Figure 3C). Oral administration of naringenin (50mg/kg and 100mg/kg body weight) and positive control glibenclamide (600µg/kg body weight) significantly (p<0.05) augmented the levels to that of control animals (Figure 3C). For instance, no significant change in GPX level was observed between control animals and the animals administered with 100mg/kg body weight naringenin or 600µg/kg body weight glibenclamide (Figure 3C). Treatment of control animals (exposed to vehicle 0.5% carboxymethyl cellulose) with 100mg/kg body weight naringenin did not change GPX levels (Figure 3C).

GST is a glutathione-dependent cytosolic enzyme, which protects cells from the damage caused by ROS. In order to determine whether administration of naringenin restore the GST activity, a method developed by Habig et al., (1974) was used and the results expressed as µmoles of CDNB-GSH conjugate formed /min/mg protein. Analysis of the data represented in Figure 3D showed a 50% decrease in GST activity, compared to vehicle treated mice, when animals were treated with STZ (Figure 3D). Oral administration of naringenin (50mg/kg and 100mg/kg body weight) and glibenclamide...
(600µg/kg b.w.) significantly (p<0.05) increased the GST activity levels. For example, administration of 100mg/kg body weight naringenin and 600µg/kg body weight glibenclimide elevated the GST levels (from ~2.9 units in STZ treated mice to ~5.6 units in glibenclimide treated mice) that are much closer to the levels observed in control animals (Figure 3D). No significant change in GST activity was observed between vehicle control and 100mg/kg body weight naringenin treated mice (Figure 3D).

GSH is potent free-radical scavenger, a co-substrate for GPX activity, and a cofactor for many redox enzymes. Several studies have shown the ability of STZ to reduce cellular glutathione levels. Therefore, to determine whether administration of naringenin could mitigate the effect of streptozotocin-induced GSH depletion effect, kidney tissue was collected from control and experimental animals and processed to estimate glutathione content using Ellman’s assay (Ellman’s et al., (1959)). The data in Figure 3E showed a significant decrease in tissue GSH levels upon treating mice with STZ (Figure 3E). However, when naringenin or glibenclimide was administered the levels had gone up to the ones present in control animals (Figure 3E). However, treatment of control animals (exposed to vehicle 0.5% carboxymethyl cellulose) with 100mg/kg body weight naringenin did not change GSH levels (Figure 3E).

DISCUSSION

Accumulating data suggests that ROS is one of the key contributors for the pathogenesis of STZ induced nephropathy. Mechanistically, STZ elevates blood glucose thereby generate excessive ROS through the incomplete oxidation of glucose. Unusually high ROS thus generated leads to serious metabolic syndromes such as diabetes. In addition, kidneys become more susceptibility to elevated glucose in blood. As a result nephropathy occurs in streptozotocin treated mice. More over, high blood glucose and excess ROS can suppresses the levels of antioxidant defense enzymes SOD, Catalase, GST and metabolites, which leads to antioxidant imbalance. Therefore, restoring normal antioxidant status is very critical to overcome the STZ-induced nephrotoxicity. Administration of naturally occurring antioxidant compounds is one way to mitigate the complications of STZ-mediated nephrotoxicity. Naringenin is a naturally occurring flavonoid, known to exhibit potent antioxidant properties. Therefore, the potential of naringenin to protect kidney tissues from STZ treatment mediated damage is investigated in this study.

As reported, MLD-STZ triggered renal damage as indicated by increase in serum urea, uric acid, creatinine, bilirubin, albumin and renal tubular changes. In addition, STZ reduced the levels of SOD, Catalase, GST and GPX in the kidney tissues. In this study we have demonstrated that treatment with 50mg/kg body weight or 100mg/kg body weight naringenin provided marked functional and histological protection against MLDSTZ induced diabetic nephropathy in mice. More over, administration of naringenin decreased the levels of TBARS and hydroperoxides in kidney tissues, implicating reduction in oxidative stress. Therefore, naringenin could be a potential candidate for developing a naturally occurring treatment agent.
for protecting kidneys from oxidative damage. However, further studies are required to elucidate key mechanism(s) by which naringenin exerts its protection.

CONCLUSION

In conclusion, the current study revealed that naringenin helps to mitigate the STZ-induced complications occurred in kidneys by promoting antioxidant defense enzymes as well as by increasing the levels of glutathione. Hence, naringenin could be used possibly as a potent anti-diabetic agent, but this require additional studies measuring and comparing its’ efficacy with known anti-diabetic agents.

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