

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* (Methylnitrosocarbonyl)- \pm -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 cancers⁴. 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 damage⁵. Flavonoids act as antioxidants to inhibit reactive oxygen species, which otherwise are detrimental to kidneys and other organs in the body⁶. Although various flavonoids such as resveratrol and hesperidin 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^{7, 8}. 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 fruits⁹⁻¹¹. Prior studies have demonstrated that naringenin could safeguard cells from the damage caused by oxidative stress and inflammatory cytokines¹². Supporting this a study showed the protection from cisplatin induced kidney damage by naringenin treatment¹³. Similarly, other studies have reported protection from doxorubicin-induced cardio-toxicity and streptozotocin (STZ) induced hepatic inflammation^{14,15}. Moreover, recently, naringenin has been reported to be a better agent compared to hesperidin (flavanones) in protecting mitochondrial and cellular membranes¹⁶. 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, Streptozotocin, 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 streptozotocin

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 days¹⁷ and blood (50 to 100 μ L) 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 diabetes¹⁸. Mice with blood glucose concentration >250mg/dL were selected for testing the potency of naringenin for mitigating STZ-induced nephrotoxicity as detailed below¹⁹. 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 cellulose². 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 puncture²¹. Serum was separated, and stored at -20 $^{\circ}$ 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 GST²².

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 method²³ and hydroperoxides was measured by the method of Jiang *et al.*, (1992)²⁴. Further, antioxidant enzymes (a) *Superoxide dismutase* by Kakkar *et al.*, (1984)²⁵; (b) *Catalase* by Sinha *et al.*, (1972)²⁶; (c) *Glutathione peroxidase* by Rotruck *et al.*, (1973)²⁷; and *Glutathione S-Transferase* by Habig *et al.*,

(1974)²⁸; and antioxidant molecules such as reduced *Glutathione* by Ellman's *et al.*, (1959)²⁹ 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 24h³⁰, 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 5¼m thickness and stained using hematoxylin-eosin³⁰. 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 bilirubin³¹. 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

Table 1: Control and experimental groups of mice:

Group	Number of mice	Category	Treatment agent	Dose and frequency of the treatment agent (mg/kg body weight)	Route of administration	Comment
I	6	Normal	Vehicle: Carboxy methyl cellulose	0.5%, Every day for 45 days	Oral	Normal vehicle control
II	6		Naringenin* (NAR)	100mg/kg, every day for 45 days	Oral	Naringenin control
III	6	STZ	Streptozotocin (STZ)	50, 5 consecutive days	Intra peritoneal	STZ control
IV	6		STZ, followed by NAR*	STZ - 50, 5 consecutive days NAR - 50mg/kg, every day for 45 days	Oral	STZ mice treated with low dose naringenin
V	6		STZ, followed by NAR*	STZ - 50, 5 consecutive days NAR - 100mg/kg, every day for 45 days	Oral	STZ mice treated with high dose naringenin
VI	6		STZ, followed by Glibenclamide*	STZ - 50, 5 consecutive days GLC - 600µg/kg, every day for 45 days	Oral	A positive control group

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)

Oral administration of naringenin retards the formation of TBARS and hydroperoxides in STZ-treated kidney tissues

Lipid peroxidation and formation of hydroperoxides are key indicators of tissue damage occurred by oxidative stress³². STZ is known to induce the levels of lipid peroxides and hydroperoxides in tissues of various organs including kidneys³³. Therefore, first, the levels of lipid peroxides were measured by boiling 0.1ml tissue homogenate with 2.0ml of TBA:TCA:HCl reagent for 15min followed by cooling, and centrifuging at 10,000rpm for 5min to separate clear supernatant. The levels of TBARS (mM / 100g tissue) were estimated by reading the absorption at 535nm. Figure 2A show a significant increase in the concentration of TBARS from about 1mM/100g tissue to ~2.5mM/100g tissue upon STZ treatment. Administration of 50 and 100mg/kg b.w. of naringenin reduced the STZ-induced TBARS to ~1.8mM/100g tissue and ~1.5mM/100g tissue, respectively. The positive control 600µg/kg body weight glibenclimide brought down the TBARS to

levels that are similar to control animals (Figure 2A). Treatment of control animals (exposed to vehicle 0.5% carboxymethyl cellulose) with 100mg/kg body weight naringenin did not change TBARS levels (Figure 2A).

To estimate the concentration of hydroperoxides 0.2ml of tissue homogenate was incubated with 1.8ml of Fox reagent at room temperature for 30minutes and the absorbance measured at 560nm. The results were expressed as mM hydroperoxides produced per 100g tissues. The bar graph in Figure 2B demonstrate that STZ-treatment significantly elevated the tissue hydroperoxides from 76mM/100g tissue to ~116mM/100 tissue. Elevated hydroperoxides were brought down to about 95mM/100g tissue and 84mM/100g tissue, respectively upon 50 and 100mg/kg b.w. naringenin administration (Figure 2B). Oral administration of glibenclamide (600µg/kg b.w.) decreased lipid peroxides to levels that are similar to control animals (Figure 2B). Treatment of control animals (exposed to vehicle 0.5% carboxymethyl cellulose) with 100mg/kg body weight naringenin did not change hydroperoxides levels (Figure 2B).

Oral administration of naringenin enhanced the activities of SOD, CAT, GSH, GPX and GST in STZ-treated kidney tissues

Increased oxidative stress in tissues modulates the expression of antioxidant defense mechanisms that include enzymes (SOD, CAT, GPX

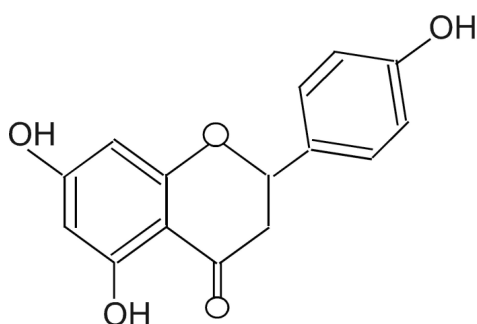
Table 2: Protective effect of naringenin on renal damage induced by streptozotocin in normal and experimental mice

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

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 H₂O₂ thereby reducing the possibility of superoxide anion interacting with nitric oxide to form reactive peroxynitrite³⁴. The SOD activity in the tissues was assayed according to the method

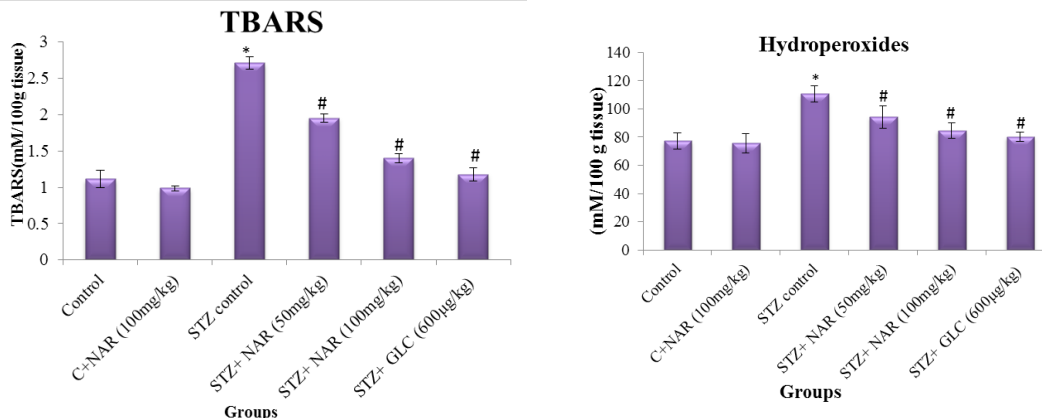
of Kakkar *et al.*, (1984)²⁵. The SOD activities were significantly ($p < 0.05$) decreased in the kidney tissue of STZ control animals (from 11.70 ± 0.1 to 6.2 ± 0.1) (Figure 3A). Oral administration of naringenin (50mg/kg and 100mg/kg body weight) and glibenclamide (600µg/kg b.w.) significantly ($p < 0.05$) 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).



Common Name: Naringenin
IUPAC Name: 5,7-Dihydroxy-2-(4-hydroxyphenyl)chroman-4-one
Molecular Weight: 272.26

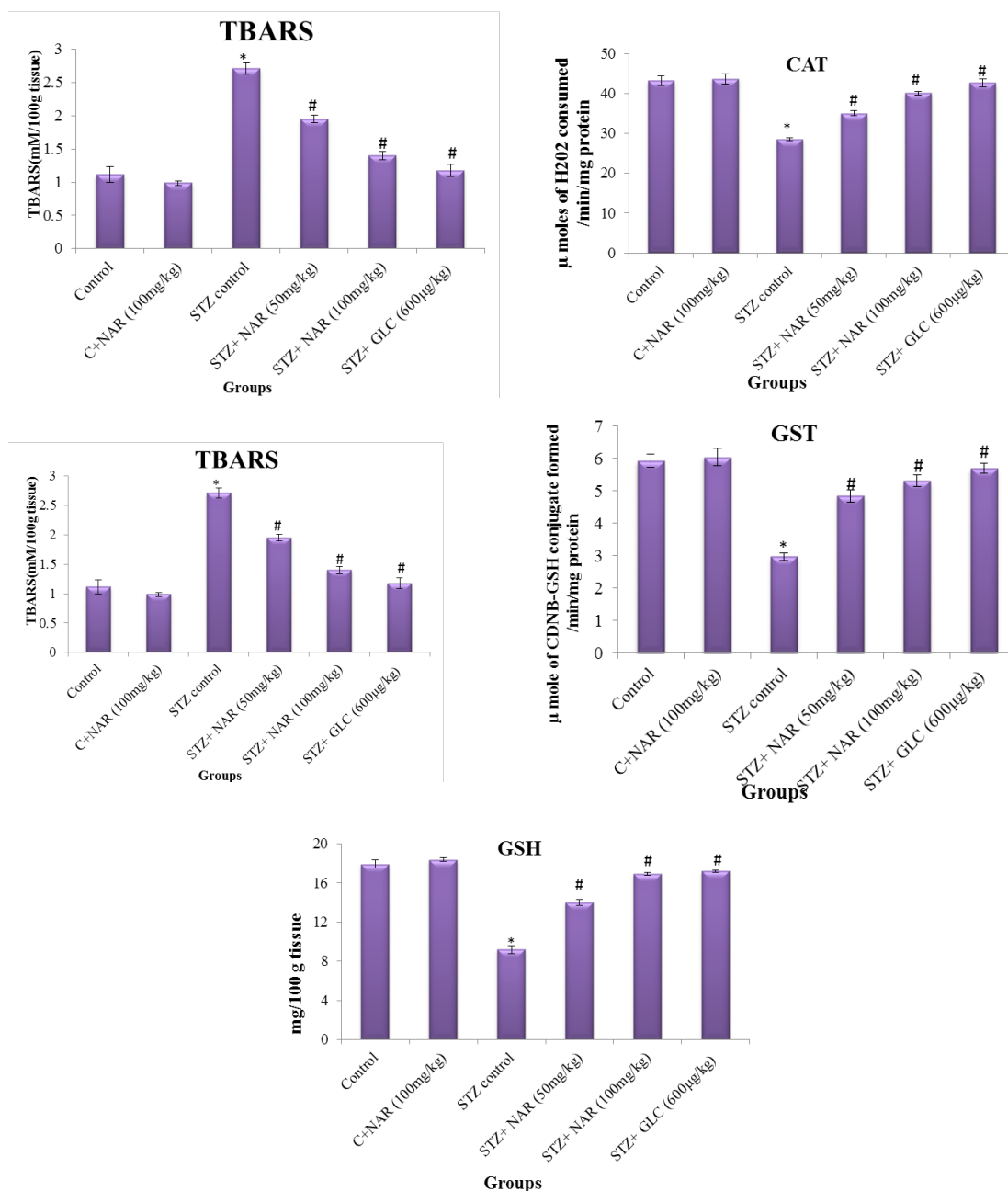
Fig. 1: Chemical structure of naringenin

Catalase is a tetrameric heme-enzyme, which decomposes hydrogen peroxide into H₂O and oxygen³⁵. The catalase activity (µmoles of H₂O₂ consumed/min/mg protein) in the tissues was assayed by the method developed by Sinha *et al.*, (1972)²⁶. 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 \pm 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. 2: Effect of naringenin on the levels of TBARS and hydroperoxides in kidney tissues of control and experimental mice



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 \pm 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 ~40units, the administration of 600µg/kg body weight glibenclimide 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).

GPX, a selenium-containing peroxidase, it decomposes H₂O₂ and lipid peroxides by using GSH, and protects the cells from free radicals³⁶. The activity of glutathione peroxidase was determined by the method of Rotruck *et al.*, (1973)²⁷. 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 glibenclimide (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 ROS³⁷. 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 protein²⁸. 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

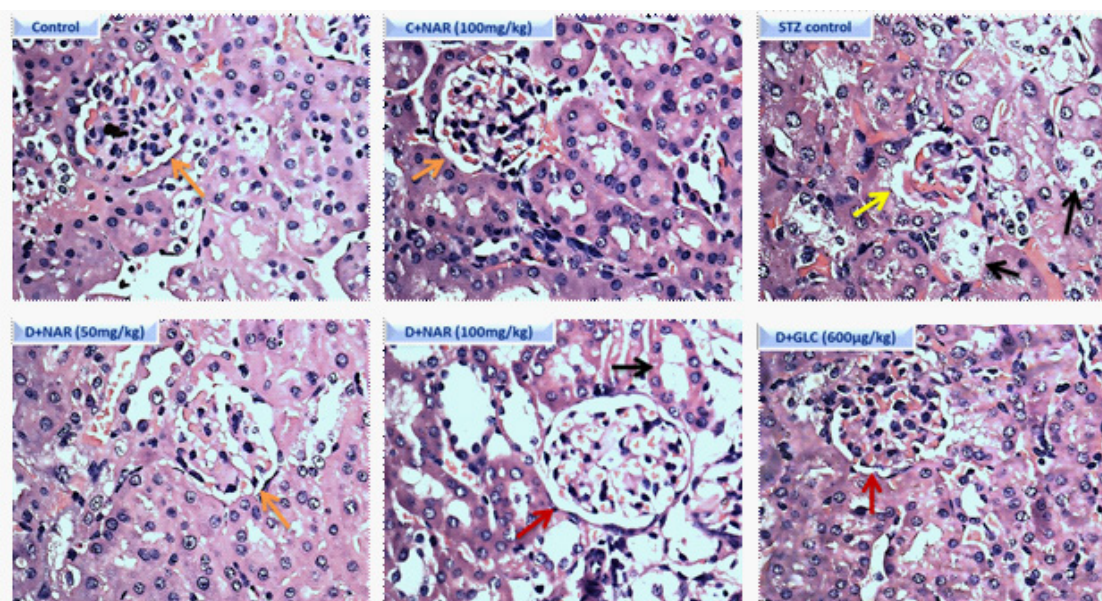


Figure 4 represents the H & E staining of kidney tissues of control and experimental mice. Control and C+NAR (100mg/kg b.w.)- Shows renal parenchyma with normal glomeruli (orange arrow) and tubules; STZ control- necrosis, bowman's space expansion (yellow arrow) and swelling of the epithelial cells; STZ+NAR (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).

Fig. 4: Histopathological investigation of kidney by H and E staining (Magnification: 40X)

(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 enzymes³⁸. 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)²⁹. 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).

Oral administration of naringenin restored the altered morphology of kidney tissues observed in mice treated with STZ

Since STZ administration is known to damage kidneys (nephrotoxicity) by reducing the size and number of functionally active glomeruli, an attempt was made to check whether naringenin and the control glibenclimide restores these altered morphological features³⁹. The data showed a well-organized renal parenchyma with normal glomeruli and tubules in control animals (Figure 4). However, kidney sections of STZ treated mice exhibited characteristic features of nephrotoxicity such as dilation of renal tubules, necrosis, bowman's space expansion and swelling of the epithelial cells (Figure 4). These changes in kidney tissue sections were mitigated by the administration of naringenin (Figure 4). While 50mg/kg body weight naringenin showed mild distortion of the histo-architecture of the kidney

with moderate necrosis, the ones received 100mg/kg body weight naringenin showed almost normal histo-architecture of the kidney with absence of fibrosis, slight congestion of glomerular tufts, and normal glomeruli (Figure 4). Kidney section of STZ mice treated with positive control compound glibenclamide showed normal histo-architecture of the kidney (Figure 4).

DISCUSSION

Accumulating data suggests that ROS is one of the key contributors for the pathogenesis of STZ induced nephropathy⁴⁰. Mechanistically, STZ elevates blood glucose thereby generate excessive ROS through the incomplete oxidation of glucose². Unusually high ROS thus generated leads to serious metabolic syndromes such as diabetes⁴¹. In addition, kidneys become more susceptibility to elevated glucose in blood⁴². As a result nephropathy occurs in streptozotocin treated mice⁴². 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 imbalance⁴³. Therefore, restoring normal antioxidant status is very critical to overcome the STZ-induced nephrotoxicity⁴⁴. Administration of naturally occurring antioxidant compounds is one way to mitigate the complications of STZ-mediated nephrotoxicity⁴⁵. 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 changes⁴⁶. In addition, STZ reduced the levels of SOD, Catalase, GST and GPX in the kidney tissues⁴⁶. 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 stress⁴⁷. 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 requires additional studies measuring and comparing its efficacy with known anti-diabetic agents

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