Nephroprotective effect of silymarin in hyperglycemia-induced oxidative stress in rats

Uma Narayanamurthy*, A. Sylvia Santhakumari, P. Nirmala

INTRODUCTION

Diabetes mellitus (DM) is a group of syndromes characterized by hyperglycemia, altered metabolism of carbohydrate, lipid and protein, resulting in microvascular and macrovascular complications. DM is one of the most common non-communicable diseases. It is the fourth or fifth leading cause of death in most high-income countries. According to Diabetes Atlas published by International Diabetes Federation, there are 382 million people living with diabetes. By 2035, this will rise to 592 million. Diabetes caused 5.1 million deaths in 2013. Diabetes imposes a large economic burden on individuals, families, national health systems and countries. Type 2 DM accounts for 85-95% of all diabetes and Type 1 DM 5-10% of all diabetes. India has more diabetes than any other country in the world, although recent data suggest that China has even more.

The American Diabetes Association has proposed a generally accepted classification of DM into three Types: type 1A associated with beta cell destruction leading to absolute deficiency of insulin is immune mediated developing in childhood or early adulthood; Type 1B (idiopathic) is a non-immune form of DM frequently seen in minorities, especially Africans and Asians, with intermittent insulin requirements; Type 2 is associated with a relative insulin deficiency and insulin resistance; Type 3 diabetes is used to encompass diabetes caused by specific genetic defects (maturity-onset diabetes of the young), infections, other endocrine abnormalities and drug-induced diabetes. Women who develop diabetes due to stress of pregnancy are classified as having gestational diabetes.

Hyperglycemia leads to increased production of reactive oxygen species (ROS). These free radicals are implicated in the complications of DM. Various animal studies have proved that hyperglycemia-induced oxidative stress causes a reduction in beta cell mass and impairment in its function. Epidemiological studies, clinical trials and...
animal experimental models have proved that dietary supplementation of antioxidants like vitamin E, vitamin C, etc., has reduced the incidence of oxidative damage related disorders such as ageing, cardiovascular diseases, diabetes, inflammation, and neurodegenerative disorder. Thus, they exert their anti-oxidant activity by various mechanisms, e.g., by scavenging or quenching free radicals, by chelating metal ions, or by inhibiting enzymatic systems responsible for free radical generation.

**Flavonoids** (more than 8000) constitute the largest and most important groups of polyphenolic compounds in fruits, vegetables, wine, tea and cocoa. Recent attention has been focused on the potential use of flavonoids-based drugs for the prevention and treatment of oxidative stress-mediated diseases. Flavonoids can exert their antioxidant activity by various mechanisms, e.g., by scavenging or quenching free radicals, by chelating metal ions, or by inhibiting enzymatic systems responsible for free radical generation.

*Silybum marianum* L. (milk thistle), a member of *Carduus marianum* family, is an ancient medicinal plant which has been used for more than 2000 years for the treatment of liver and gall bladder disorders. Silymarin is the active component of this herb that is a mixture of four flavonolignans namely silybin, isosilybin, silydianin and silychristin. Recently silymarin has received attention due to its beneficial effects such as anticancer, chemoprotective, hypcholesterolemic, cardioprotective, neuroactive, neuroprotective and skin protective activities. Besides the cytoprotective activity of silymarin mediated by its anti-oxidative and radical scavenging properties also new activities based on specific receptor interaction were discovered. These include stimulation of ribosomal RNA polymerase, and subsequent protein synthesis, leading to enhanced hepatocyte regeneration, pro-apoptotic and anti-angiogenic activity, inhibition of nuclear factor kappa B activity, inhibition of epidermal growth factor receptor – mitogen-activated protein kinase/extracellular regulated kinases ½ signaling, modulation of P-glycoproteins, etc. Few animal studies have proved the anti-diabetic activity of silymarin. Hence, the present study was done to explore the nephroprotective effect of silymarin against hyperglycemia-induced oxidative stress in diabetic rats.

**METHODS**

**Study center**

This study was undertaken at Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar. All studies were conducted in accordance with the National Institute of Health “Guide for the care and use of Laboratory Animals” (NIH, 1985). The study was approved by the Animal Ethical Committee of Rajah Muthiah Medical College and Hospital (Registration No. 160/1999/ [CPCSEA]) Annamalai University, Annamalai Nagar, Tamil Nadu, India (Proposal No.1017, dated 02-05-2013).

**Materials**

**Chemicals and reagents**

Silymarin (milk thistle) was purchased from Nature’s Bounty Inc., Bohemia, NY 11716 U.S.A. Alloxan monohydrate (2,4,5,6-tetraoxyxypirimidine-2,4,5,6-pyrimidinetrione) was purchased from MP Biomedicals India Private Limited, Mumbai, Maharashtra. Biomedical and enzymatic kits for measuring lipid peroxidase and antioxidants enzymes were obtained from Sigma-Aldrich Chemicals and Mouli Enterprises. Blood glucose was determined using glucometer (strip test in one touch), obtained from Ramesh Surgical and Enterprises, Madurai. Serum urea and creatinine were determined by biochemical analyzer using commercial kits, obtained from Sigma-Aldrich Chemicals, Chennai.

**Preparation of drug**

Silymarin powder was dissolved in distilled water to make a solution of 50 mg/ml. Glibenclamide tablets were dissolved in distilled water to make a solution of 0.25 mg/ml.

**Animals**

Healthy adult male rats of Wistar strain weighing 230-250 g were used in the present study. They were purchased from the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar, Tamil Nadu, India. Animals were housed in polypropylene cages (28 cm × 22 cm × 14 cm) bedded with husk in groups of six under controlled environmental conditions (temperature - 23±2°C, humidity 65-70% and 12 hrs light/dark cycles) at Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. Animals were fed with standard pellet diet (VRK Nutritional Solutions, Baramati Agro Limited, Sangli, Maharashtra, India) and water ad libitum.

**Induction of DM**

Alloxan monohydrate powder was dissolved in distilled water to make a solution of 50 mg/ml. The rats were fasted overnight, and hyperglycemia was induced by single SC injection of alloxan monohydrate (100 mg/kg). The rats were maintained on 5% glucose solution for next 24 hrs to prevent hypoglycemia. The animals had access to food and water. The development of hyperglycemia in rats was confirmed by estimating fasting blood glucose at 48 hrs after alloxan monohydrate injection. The rats with fasting blood glucose level >150 mg/dl were considered as diabetic and were included in the study.

**Study design**

The rats were divided into five groups of six each (n=6): Group I (n=6) normal control received 1 ml distilled water.
Group II (n=6) diabetic rats received 1 ml distilled water.
Group III (n=6) diabetic rats received silymarin 200 mg/kg (PO).
Group IV (n=6) diabetic rats received silymarin 400 mg/kg (PO).
Group V (n=6) diabetic rats received glibenclamide 0.5 mg/kg (PO).

The drug treatment was carried out everyday morning using intragastric tube for 3 weeks after induction of DM.

**Blood sampling**

At the end of 7, 14 and 21 days of inducing DM fasting blood glucose levels were estimated in all the five groups using SD Code free glucometer. Blood samples were gathered by tail snipping method. On the 21st day of the experiment, blood samples were taken by retro-orbital puncture under intramuscular ketamine from all the groups of rats for biochemical analysis.

**Serum preparation**

The whole blood was collected from rats of each group in sterile, covered test tubes and labeled. After collection of the whole blood, it was allowed to clot undisturbed for 15-30 mins. The clot was removed by centrifuging at 1000-2000 ×g for 10 mins in a centrifuge. The supernatant serum was obtained for biochemical analysis.

**Tissue sampling**

The animals were sacrificed by cervical dislocation and the kidneys from all the groups of rats were dissected out. They were processed for histopathological examination (HPE) and biochemical analysis.

**Light microscopic study**

For histopathological study the rat tissues were perfused with 10% formalin. The kidneys of the rats were excised immediately from the abdominal cavity and fixed in 10% neutral formalin, dehydrated in graded alcohol (80-100%), cleaned in xylene and embedded in paraffin. Then renal tissues were sliced into 3-5 μm pieces with a rotary microtome, deparaffinized in xylene, passed through varying grades of alcohol and finally stained with hematoxylin and eosin for histopathological assessment. The specimens were evaluated with a light microscope. All histopathological changes were examined by pathologist.

**Biochemical analysis**

Blood urea was estimated by urease method and serum creatinine was estimated by modified Jaffe’s method. Reduced glutathione in the tissues was estimated by the method of Niehaus and Samuelsson. Reduced glutathione in the tissues were estimated by the method of Ellman. Superoxide dismutase (SOD) in the tissues was assayed by the method of Kakkar et al., and the activity of catalase (CAT) in the tissues were determined by the method of Sinha.

**Statistical analysis**

Values of biochemical analysis were expressed as means±standard deviation for six rats in each group. The data were analyzed by Duncan’s Multiple Range Test, using SPSS software version 17.0 (SPSS, Inc., Chicago, Illinois). Values not sharing a common superscript differ significantly at p<0.05.

**RESULTS**

**Effect of silymarin on blood glucose levels**

The glucose values were compared to values obtained for diabetic control rats and silymarin treated rats. In alloxan treated diabetic rats, the blood glucose levels remained significantly high on all the 3 weeks. Silymarin treated rats showed a significant reduction in blood glucose levels from 2nd week after induction. Silymarin treated rats showed maximum reduction in blood glucose level on 21st day at 400 mg/kg. In glibenclamide treated rats, blood glucose levels were significantly lower in all the 3 weeks compared to silymarin treated rats (Table 1).

**Effect of silymarin on lipid peroxides (LPO) and antioxidants in kidneys**

From this study it was observed that LPO level was significantly higher in diabetic rats compared to normal control rats. The diabetic rats treated with silymarin showed significant reduction in blood glucose levels from 2nd week after induction. Silymarin treated rats showed maximum reduction in blood glucose level on 21st day at 400 mg/kg. In glibenclamide treated rats, blood glucose levels were significantly lower in all the 3 weeks compared to silymarin treated rats. Glibenclamide treated rats also showed significant increase in antioxidant levels compared with diabetic rats (Table 2).

**Effect of silymarin on serum urea and creatinine**

The serum urea and creatinine levels were increased significantly in alloxan treated diabetic rats, compared to normal control rats at the end of 3rd week. The rats treated with silymarin after induction of diabetes showed significant (p<0.05) reduction in serum urea and creatinine in a dose-dependent manner compared with untreated diabetic rats. Glibenclamide treated rats also showed significant (p<0.05) reduction in serum urea and creatinine compared with diabetic rats (Table 3).
atrophied renal corpuscle and mild glomerular capillaries congestion (Figure 5).

DISCUSSION

The findings of the Diabetic Complications and Control Trial (DCCT) and The United Kingdom Prospective Diabetic Study support the idea that chronic hyperglycemia plays a causative role in the pathogenesis of diabetic micro and macrovascular complications. Diabetic nephropathy is the leading contributor to end-stage renal disease. Hyperglycemia leads to increased production of ROS or superoxide in the mitochondria from glucose auto-oxidation, protein glycosylation and glucose metabolism via sorbital pathway. The ROS and glycosylation lead to disruption of cellular function, oxidative damage to

Table 1: Effect of silymarin on blood glucose levels in diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 1 (mg/dl)</th>
<th>Day 7 (mg/dl)</th>
<th>Day 14 (mg/dl)</th>
<th>Day 21 (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>76.00±3.35a</td>
<td>76.00±3.35a</td>
<td>76.00±3.35a</td>
<td>76.00±3.35a</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>202.33±2.58b</td>
<td>184.83±4.83a</td>
<td>201.17±9.52a</td>
<td>206.00±10.95a</td>
</tr>
<tr>
<td>Diabetes+silymarin (200 mg/kg)</td>
<td>200.83±8.72b</td>
<td>176.67±4.13b</td>
<td>157.50±9.38b</td>
<td>127.33±1.63b</td>
</tr>
<tr>
<td>Diabetes+silymarin (400 mg/kg)</td>
<td>208.33±11.3b</td>
<td>171.17±2.99b</td>
<td>143.83±6.21b</td>
<td>118.67±3.01b</td>
</tr>
<tr>
<td>Diabetes+silymarin+glibenclamide (0.5 mg/kg)</td>
<td>199.67±5.13c</td>
<td>155.33±3.44d</td>
<td>131.17±6.14d</td>
<td>108.83±2.04d</td>
</tr>
</tbody>
</table>

Values are expressed as means±SD for six rats in each group. Values not sharing a common superscript differ significantly at p≤0.05 (Dunken’s test). SD: Standard deviation

Table 2: Effect of silymarin on LPO and antioxidants in kidneys of diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (unit/mg protein)</th>
<th>GSH (µg/mg protein)</th>
<th>CAT (µmol/mg protein)</th>
<th>LPO (mmole/100 g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>15.90±0.35a</td>
<td>31.72±0.86b</td>
<td>19.51±0.97a</td>
<td>3.55±0.36d</td>
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<tr>
<td>Diabetic control</td>
<td>5.11±0.58c</td>
<td>8.10±0.21c</td>
<td>3.38±0.48e</td>
<td>16.59±0.37c</td>
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<tr>
<td>Diabetes+silymarin (200 mg/kg)</td>
<td>11.12±0.71d</td>
<td>20.39±0.43d</td>
<td>17.92±0.72e</td>
<td>8.72±0.90c</td>
</tr>
<tr>
<td>Diabetes+silymarin (400 mg/kg)</td>
<td>15.30±0.46b</td>
<td>30.57±0.78c</td>
<td>17.72±0.66c</td>
<td>8.19±0.45c</td>
</tr>
<tr>
<td>Diabetes+silymarin+glibenclamide (0.5 mg/kg)</td>
<td>13.86±0.12c</td>
<td>31.97±1.18b</td>
<td>18.24±0.64c</td>
<td>3.42±0.72e</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD for six rats in each group. Values not sharing a common superscript differ significantly at p≤0.05 (Dunken’s test). SD: Standard deviation, LPO: Lipid peroxides, SOD: Superoxide dismutase, GSH: Glutathione, CAT: Catalase

Table 3: Effect of silymarin on serum urea and serum creatinine in diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Urea (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>26.92±1.77e</td>
<td>0.58±0.05e</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>75.13±4.29a</td>
<td>1.78±0.09a</td>
</tr>
<tr>
<td>Diabetes+silymarin (200 mg/kg)</td>
<td>49.46±0.99b</td>
<td>1.65±0.11b</td>
</tr>
<tr>
<td>Diabetes+silymarin (400 mg/kg)</td>
<td>31.30±0.56d</td>
<td>0.64±0.04d</td>
</tr>
<tr>
<td>Diabetes+glibenclamide (0.5 mg/kg)</td>
<td>31.40±0.77e</td>
<td>0.72±0.02e</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD for six rats in each group. Values not sharing a common superscript differ significantly at p≤0.05 (Dunken’s test). SD: Standard deviation

Histopathological study of renal cortex

Compared to the photomicrograph of renal cortex of normal rats (Figure 1), the diabetic rats showed renal cellular injury, necrotic tubular epithelium with widening of Bowman’s capsule and the glomerular atrophy (Figure 2).

Diabetic rats treated with silymarin 200 mg/kg showed severe glomerular atrophy and necrosis of tubular epithelial cells (Figure 3).

However at silymarin 400 mg/kg, the renal cortex showed only mild swelling and congestion in the renal epithelium. There was no necrosis and the glomerular atrophy (Figure 4).

In diabetic rats treated with glibenclamide, the renal cortex showed moderate degeneration of renal epithelium, atrophied renal corpuscle and mild glomerular capillaries congestion (Figure 5).
the membrane and other structures, susceptibility to LPO and exhaustion of antioxidant defences. Al-Enazi had reported rise in LPO in hepatic cells due to hyperglycemia induced by streptozotocin in rats. In the present study also the diabetic rats showed an increase in LPO and decreased in antioxidants in the renal tissue. The oxidative damage to renal cell was further confirmed by increase in serum urea and creatinine in diabetic rats.

There are reports that natural antioxidants such as vitamin E, caffeic acid, lipoic acid, quercetin, melatonin and natural phenolic compounds have protective effects against hyperglycemia-induced oxidative stress. Treatment of diabetic animals with silymarin significantly reduced LPO and increased the levels of SOD, glutathione reductase and CAT. SOD protects tissues against oxygen free radicals by scavenging $O_2^-$. Thus, SOD can act as a primary defense against $O_2^-$ and prevents further generation of free radicals (Arivazhagan et al., 2000). CAT protects tissues from highly reactive OH radicals (Sozmen et al., 2001). Silymarin by scavenging the free radicals and by raising the antioxidant activities prevented the renal damage and maintained the cell integrity. The DCCT demonstrated that the improvement of glycemic control reduced microalbuminuria (39%) and clinical nephropathy (54%). Varzi et al. had reported that silymarin improved alteration in serum creatinine concentration in gentamicin treated dogs. In the present study also silymarin treated diabetic animals showed a decrease in serum urea and creatinine, which confirms its nephroprotective effect. The cytoprotective action of silymarin against hyperglycemia-induced oxidative stress...
was further revealed by HPE study. The histopathological study of renal tissue of diabetic rats showed the glomerular atrophy with necrosis of tubular epithelium. This damage was due to hyperglycemia. In silymarin treated diabetic rats there was restoration of glomerular structure, with only mild congestion in tubular epithelium.

CONCLUSION
This study proved that the silymarin had significant nephroprotective effect against the hyperglycemia-induced oxidative stress. Its good safety profile, easy availability and low cost are added advantages. Hence, silymarin may be added as an adjuvant therapy for preventing or slowing the progression of diabetic nephropyathy.

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Ethical Approval: This study was approved by the Institutional Animal Ethics Committee [160/1999/(CPCSEA)]

REFERENCES