Anti-hyperglycemic activity of Rutin in streptozotocin-induced diabetic rats: An effect mediated through cytokines, antioxidants and lipid biomarkers

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Administration of rutin (50 and 100 mg/kg) and pioglitazone (10 mg/kg) orally for 3 weeks treatment significantly improved body weight, reduced plasma glucose and glycosylated hemoglobin, pro-inflammatory cytokines (IL-6 and TNF-α), restored the depleted liver antioxidant status and serum lipid profile in high fat diet + streptozotocin induced type 2 diabetic rats. Rutin treatment also improved histo-architecture of β islets and reversed hypertrophy of hepatocytes. Rutin exhibited significant antidiabetic activity, presumably by inhibiting inflammatory cytokines, improving antioxidant and plasma lipid profiles in High fat diet + streptozotocin induced type 2 diabetic model and may be useful as a diabetic modulator along with standard antidiabetic drugs. However, such effects need to be confirmed on human subjects in clinical condition.

Keywords: Antidiabetic activity, Antioxidants, Lipid profile, Pro-inflammatory cytokines, Rutin, Streptozotocin

Diabetes is defined as a state in which the homeostasis of carbohydrate and lipid metabolism is improperly regulated by the insulin, ultimately leading to plasma glucose elevation. It is the world’s largest endocrine disorder, and is one of the major killers in recent times¹. Therefore, it is necessary to search for new drugs and interventions that are useful in the management of this metabolic disorder. The most prevalent form of diabetes is non-insulin dependent diabetes; type 2 diabetes mellitus (type 2 DM). Rats fed with high fat diet (HFD) and then followed by streptozotocin (STZ) injection result in hyperglycemia, hyperlipidemia and increased body weight, which is considered to be a clinically ideal alternative animal model for anti-diabetic drug evaluation². Epidemiological, clinical and experimental studies have indicated a relationship between oxidative stress, low-grade inflammation in the development of type 2 DM and also its late stage complications³. In type 2 DM, the production of reactive oxygen species (ROS) are increased due to insulin resistance and hyperglycemia⁴. The elevated glucose is itself pro-inflammatory and increased the level of acute-phase inflammatory markers, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and C-reactive protein (CRP). Such acute-phase inflammatory markers are found to be associated with insulin resistance and metabolic syndrome, suggesting a specific role for chronic low-grade inflammation in type 2 DM⁵, ⁶. Herbs and phytochemicals are known to play a major role in the discovery of new therapeutic agents, and have received much attention as sources of biologically active substances including antioxidants, hypoglycemic and hypolipidemic agents⁷. Flavonoids and polyphenols are being used to treat diabetes and dyslipidemia⁸. This is based on the fact that, excessive oxidative stress is implicated in the pathology and complications of DM. Further, it is documented that polyphenols with antioxidant properties exert beneficial antidiabetic effect by correcting the disturbed oxidative milieu in diabetic conditions⁹. Rutin is the glycoside between the flavonols quercetin and the disaccharide, rutinose. Rutin exerts antidiabetic, antithrombotic, anti-inflammatory, antioxidant, anticarcinogenic, cytoprotective, hepatoprotective, vasoprotective, smooth muscle relaxing, and tissue protein glycation inhibiting activities¹⁰-¹³. Its antioxidant activity is responsible for many of its biological/pharmacological effects⁹-¹¹. Keeping such documented reports in view, the present study has been undertaken to evaluate
anti-hyperglycemic activity of rutin in streptozotocin induced diabetic rats along with its effect on proinflammatory markers (TNF-α and IL-6), antioxidants and lipid biomarkers. Further, such investigation on rutin in diabetes may facilitate to explore the role of phytochemicals of antioxidant nature (polyphenols, flavonoids and others) as an adjuvant or modulators in diabetes, either per se or along with other antidiabetic drugs; in clinical medicine to prevent pro inflammatory mediators as well as oxidative stress derived tissue or organ injuries.

Materials and Methods

Chemicals and reagents—Pioglitazone HCl (Cipla Pharmaceuticals Ltd, India), rutin (Sigma Aldrich, USA), streptozotocin (Sigma-Aldrich, USA), malonaldehyde (MDA), superoxide dismutase (SOD), catalase (CAT) (Sigma-Aldrich, USA), reduced glutathione (Loba Chem, India), rat TNF-α and IL-6 ELISA kits (Thermo Scientific, USA), lipid kits (Span diagnostics, India) for triglycerides, total cholesterol, high density lipoprotein assay, were procured. Other chemicals used in the study were of analytical grade and purchased locally.

Animals—Sprague Dawley rats of either sex (160-180 g) were placed separately in clean polypropylene cages randomly with paddy husk as bedding. The animals were maintained under standard laboratory conditions (24±2 °C, 45±10% RH and 12:12 h L:D cycles) and fed with standard animal diet food pellet (Nutrivet Lab. Pune, Maharashtra) and water ad libitum. The animals were shifted to experimental laboratory 1 h prior to the experiments. All the experimental procedures and protocols of the study were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Sinhgad College of Pharmacy, Pune (SCOP/IAEC/2010-11/12), and conform to the Indian National Science Academy guidelines for the use and care of experimental animals in research.

Establishment of high fat diet (HFD) fed and STZ-treated type 2 diabetes in rats—Normal pellet diet (NPD) consists of 5% fat, 21% protein, 65% carbohydrate, 5% fibers, 4% minerals, as a percentage of total kcal and HFD (Table 1) consist of 58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal14. The composition and preparation of HFD was as per Srinivasan et al14. Animals were maintained on NPD/HFD for 4 weeks, and various biochemical parameters [body weight, plasma glucose level, plasma total cholesterol (PTC), plasma triglyceride (PTG), plasma high density lipoprotein (PHDL), plasma low density lipoprotein (PLDL), plasma very low density lipoprotein (PVLDL)] were studied before and after 4 weeks. After 4 weeks of dietary manipulations rats were administered streptozotocin (35 mg/kg, ip). Streptozotocin was prepared in cold citrate buffer (pH 4.4, 0.1 M) and administered to overnight fasted rats. Control rats were injected with cold citrate buffer (pH 4.4, 0.1 M) ip. The rats after attaining the stable plasma glucose ≥300 mg/dL following streptozotocin treatment span of 4 days were considered diabetic and selected for further studies. The rats were allowed to continue to feed on their respective diets until the end of the study.

Experimental design—A total of 30 (6 normal; 24 HFD + STZ induced diabetic rats) were randomly divided in to 5 groups (6 rats/group) and received following treatment:

| Gr-I: Served as normal control received vehicle (5 mL/kg, po) daily for 3 weeks (NC) |
| Gr-II: Served as diabetic control received vehicle (5 mL/kg, po) daily for 3 weeks (DC) |
| Gr-III: Diabetic rats + pioglitazone (10 mg/kg, po) daily for 3 weeks (DC + pio) |
| Gr- IV: Diabetic rats + rutin (50 mg/kg, po) daily for 3 weeks (DC + rutin) |
| Gr- V: Diabetic rats + rutin (100 mg/kg, po) daily for 3 weeks (DC + rutin) |

Table 1—Composition of high fat diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powdered NPD</td>
<td>365</td>
</tr>
<tr>
<td>Lard</td>
<td>310</td>
</tr>
<tr>
<td>Casein</td>
<td>250</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10</td>
</tr>
<tr>
<td>Vitamin and mineral mix</td>
<td>60</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>03</td>
</tr>
<tr>
<td>Yeast powder</td>
<td>01</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>01</td>
</tr>
</tbody>
</table>
At the end of 3 weeks, rats were anesthetized with diethyl ether and blood samples were collected directly from heart and then sacrificed by cervical dislocation. Pancreas and liver were removed and washed with cold saline solution and preserved for histopathological and biochemical studies. Body weights of rats were recorded using a digital weighing balance prior to sacrifice.

**Preparation of rutin and pioglitazone**—A homogenous suspension of rutin and pioglitazone was prepared using 1% (w/v) carboxy methyl cellulose (CMC). The suspension was prepared freshly prior to administration, and administered daily between 10.00-11.00 hrs for 3 weeks.

**Estimation of plasma glucose and glycosylated haemoglobin**—The plasma glucose was assayed by the glucose oxidase/peroxidase (GOD/POD) method using a standard reagent kit obtained from Span Diagnostics, India and the glycosylated hemoglobin as per Alayash et al.

**Insulin resistance**—Conversion of glucose in mg/dL to mmol/L was calculated by dividing glucose by 18 (constant) and values greater than 2 were indicative of insulin resistance.

**Determination of cytokines**—Tumor necrosis factor (TNF-α) and interlukin-6 (IL-6) in serum levels were assayed by enzyme-linked immunosorbent assay (ELISA) using ready made kit reagents supplied by Thermo Scientific (USA).

**Antioxidants assay in liver homogenates**—Livers were quickly excised and made free from adhering tissues, washed and perfused with chilled normal saline, minced, homogenized in ice bath using homogenizer (Biolab) in chilled 0.15 M KCl to obtain 10% (w/v) liver homogenates. Homogenates were centrifuged at 800 rpm for 10 min. The absorbance of the organic layer was read at 532 nm. MDA levels were calculated using standard curve of malondialdehyde and its content expressed in nmol/mg of protein.

**Glutathione (GSH) assay**—To 1 mL of liver supernatant, 1.8 mL of water and 2 mL of phosphate buffer (pH 7) were added. After 5 min 200 µL of DTNB reagent was added to the reaction mixture and absorbance was read at 412 nm. The amount of glutathione was determined using standard curve of glutathione and its level expressed in terms of μmol/mg of protein.

**Superoxides (SOD) assay**—To 100 µL of liver supernant, 1 mL of sodium carbonate (1.06 gm in 100 mL water), 0.4 mL of 24 mM nitroblue tetrazolium (NBT) and 0.2 mL of EDTA (37 mg in 100 mL water) was added and zero minute reading was taken at 560 nm. Reaction was initiated by addition of 0.4 mL of 1 mM hydroxylamine hydrochloride, incubated at 25 °C for 5 mins and the reduction of NBT was measured at 560 nm. SOD level was calculated using standard calibration curve and expressed in U/mg of protein.

**Catalase (CAT) assay**—Briefly, the assay mixture consisted of 0.05 M phosphate buffer (pH 7.0), 0.019 M H₂O₂ and 0.05 mL phenylmethylsulfonylfluoride (PMSF) in a total volume of 3.0 mL.

Changes in absorbance were recorded at 240 nm. Catalase activity was expressed as nmol Hydrogen peroxide consumed min/mg/protein.

**Protein determination**—The levels of total protein were determined in liver homogenates.

**Assay of lipids**—Plasma triglycerides (TG), total cholesterol (TC) and high density lipoproteins (HDL) were assayed using standard kits obtained from Span Diagnostics, India. Very low-density lipoproteins (VLDL) and low density lipoproteins (LDL) of plasma were calculated by the following formula:

\[ \text{VLDL = TG/5, LDL = TC} - (\text{HDL + VLDL}) \]

**Histopathology of pancreas and liver**—Portion each of the pancreatic and liver tissues were fixed in 10% neutral formal saline for histological studies. After fixation, tissues were embedded in paraffin; solid sections were cut at 3-4 μm thickness and stained with eosin and haemotoxylin. The sections were examined under light microscope at a magnification of 40X and photomicrographs were taken.

**Statistical analysis**—The mean ± SE values were calculated for each group. All data were subjected to one way ANOVA followed by Dunnett’s test. P values < 0.05 were considered statistically significant.
**Results**

The mean body weight and plasma lipids of rats during dietary manipulation were altered in streptozotocin induced diabetic condition, and with the treatment of rutin and pioglitazone, restored significantly (Tables 2 and 3).

A significant increase in plasma glucose and glycosylated hemoglobin was observed in diabetic (STZ + HFD) rats and treatment with rutin and pioglitazone decreased both plasma glucose and glycosylated hemoglobin significantly (Fig.1a and b).

Serum TNF-α and IL-6 were increased significantly in diabetic (STZ + HFD) rats and treatment with rutin and pioglitazone decreased the increased serum TNF-α and IL-6 significantly (Fig.1c and d).

The elevation of liver MDA in the diabetic (HFD + STZ) rats was significantly prevented with the treatment of rutin and pioglitazone (Table 4). Liver anti-oxidant enzymes GSH, SOD and CAT in the diabetic (HFD + STZ) rats were depleted. The treatment of rutin and pioglitazone restored the depleted anti-oxidant enzymes significantly (Table 4).

The elevated plasma TC, TG, VLDL and LDL and decreased HDL in diabetic (STZ + HFD) rats were reversed with the treatment of rutin and pioglitazone. (Fig. 2a-e).

Examination of histological sections of pancreas of diabetic rats showed shrinkage both in size and number of β-islets (Fig. 3A and 4B). Rutin and pioglitazone improved the histoarchitecture of the β-islets (Fig. 3C, D, E). The histopathology of diabetic rat liver showed hypertrophy of hepatocytes and increased infiltration of lymphocytes (Fig. 3B). Treatment with rutin and pioglitazone reversed histological changes in the liver (Fig. 3C, D, E) to

**Table 2—Effect of NPD and HFD on plasma parameters after 4 weeks of dietary manipulations**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body weight (g)</th>
<th>PG (mg/dL)</th>
<th>PTC (mg/dL)</th>
<th>PTG (mg/dL)</th>
<th>PHDL (mg/dL)</th>
<th>PVLDL (mg/dL)</th>
<th>PLDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPD</td>
<td>212.0 ± 3.74</td>
<td>78.88 ± 2.87</td>
<td>88.82 ± 9.00</td>
<td>76.04 ± 2.40</td>
<td>44.92 ± 2.87</td>
<td>15.21 ± 0.42</td>
<td>28.08 ± 5.43</td>
</tr>
<tr>
<td>HFD</td>
<td>232.0 ± 3.74</td>
<td>116.8 ± 3.17</td>
<td>121.7 ± 4.90</td>
<td>100.7 ± 3.44</td>
<td>38.58 ± 0.50</td>
<td>20.15 ± 0.30</td>
<td>67.03 ± 5.22</td>
</tr>
</tbody>
</table>

PG = plasma glucose, PTC = plasma total cholesterol, PTG = plasma triglyceride, PHDL = high density lipoproteins, PVLDL = plasma very low density lipoproteins, PLDL = plasma low density lipoproteins

**Table 3—Effect of rutin (3 weeks treatment) on body weight (g) of HFD+STZ-induced diabetes in rats**

<table>
<thead>
<tr>
<th>Group and treatment</th>
<th>After HFD</th>
<th>After STZ</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Normal Control (NC)</td>
<td>212.00 ± 3.74</td>
<td>216.00 ± 2.44</td>
<td>228.00 ± 4.89</td>
</tr>
<tr>
<td>II Diabetic Control (DC)</td>
<td>232.00 ± 3.74</td>
<td>218.00 ± 3.74</td>
<td>184.00 ± 4.00</td>
</tr>
<tr>
<td>III DC + Pio (10)</td>
<td>236.00 ± 5.09</td>
<td>224.00 ± 5.09</td>
<td>240.00 ± 3.16</td>
</tr>
<tr>
<td>IV DC + rutin (50)</td>
<td>236.00 ± 7.48</td>
<td>220.00 ± 4.47</td>
<td>240.00 ± 4.47</td>
</tr>
<tr>
<td>V DC + rutin (100)</td>
<td>236.00 ± 6.00</td>
<td>222.00 ± 5.83</td>
<td>242.00 ± 5.83</td>
</tr>
</tbody>
</table>

P < 0.05; compared to *normal control, *diabetic control
near normal and decreased the infiltration of lymphocytes (Fig. 3F-J).

Discussion
In the present study, HFD-fed rats showed slightly hyperglycemia and were more susceptible to diabetogenic conditions (hyperglycemic and hyperlipidemic). These rats on treatment with STZ become diabetic that resembles to human type 2 DM. The HFD + STZ induced diabetic rat model has been used widely for type 2 DM studies. HFD + STZ induced diabetic rats when treated with rutin (50 and 100 mg/kg) elicited a dose related hypoglycemic effect.

In recent years, several researchers have studied the efficacy of different medicinal plants/herbs in modulating the disturbed inflammatory markers, redox status and lipid related alterations in type 2 DM. During the progression of diabetes the excess glucose present in blood reacts with hemoglobin and

Table 4—Effect of rutin (3 weeks treatment) on liver MDA, GSH, SOD and CAT of HFD + STZ-induced diabetes in rats
[Values are mean ± SE from 6 rats in each group]

<table>
<thead>
<tr>
<th>Group and treatment (mg/kg, po)</th>
<th>MDA (nmol/mg protein)</th>
<th>GSH (µmol/mg protein)</th>
<th>SOD (U/mg protein)</th>
<th>CAT (nmol H₂O₂/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Normal control (NC)</td>
<td>1.13± 0.07</td>
<td>21.20± 1.28</td>
<td>84.34± 5.20</td>
<td>179.30± 6.50</td>
</tr>
<tr>
<td>II Diabetic control (DC)</td>
<td>2.94± 0.27*</td>
<td>13.60± 1.16*</td>
<td>37.94±2.96*</td>
<td>90.88± 4.55*</td>
</tr>
<tr>
<td>III DC + pio (10)</td>
<td>1.80± 0.05*</td>
<td>17.00± 1.51*</td>
<td>63.74± 4.48*</td>
<td>133.30± 3.76*</td>
</tr>
<tr>
<td>IV DC + rutin (50)</td>
<td>1.56± 0.09*</td>
<td>17.60± 0.74*</td>
<td>68.68 ±2.70</td>
<td>131.50± 5.61</td>
</tr>
<tr>
<td>V DC + rutin (100)</td>
<td>1.43± 0.04*</td>
<td>19.20± 1.06*</td>
<td>74.78± 2.60*</td>
<td>139.90± 3.16*</td>
</tr>
</tbody>
</table>

* P < 0.05; compared to "normal control, *diabetic control.

Fig. 2—Effect of 3 weeks treatment of rutin on serum (a) plasma total cholesterol (b) plasma triglyceride (c) VLDL (d) plasma HDL (e) plasma LDL of HFD + STZ induced diabetes in rats. P<0.05 compared to @normal control, *diabetic control.
is elevated to nearly two folds above the normal level in HFD-diabetic rats. Treatment with rutin decreased plasma glucose, increased body weight and decreased glycosylated hemoglobin significantly, and its effect was comparable to that of pioglitazone, a clinically effective new generation anti-diabetic drug.

Cytokines, TNF-α and IL-6 are intimately associated with insulin resistance, a condition of low insulin sensitivity. During type 2 DM, the ability of insulin to lower blood glucose is impaired, causing metabolic syndrome condition. TNF-α elicits antagonistic activity towards insulin because of its ability to augment insulin receptor substrate (IRS) phosphorylation on serine or threonine residues. Altered IRS phosphorylation on serine or threonine reduces the phosphorylation of tyrosine residues through protein kinase C and the nuclear factor kappaB (NF-κb), a regulatory protein kinase (Iκκβ). The reduction in tyrosine phosphorylation causes insulin resistance. The inhibition of intracellular signaling of insulin mediated through TNF-α resulted in the suppression of the regulatory enzymes of fatty acids and also glucose capture, including the synthesis of triglycerides that finally results into hyperglycemia.

IL-6 is also considered to be a pro-inflammatory cytokine that is known to cause insulin resistance in skeletal muscles as well as in liver due to the defects in IRS phosphorylation resulting in decreased gluconeogenesis and increased glycogenolysis. The reduction in intracellular pro-inflammatory mediators by rutin treatments might have improved IRS-phosphorylation. Such improvement of cellular event enables to reverse the inhibitory effect on insulin signaling pathways, mainly the enzymes of fatty acids, glucose uptake process and depletion of triglyceride synthesis.

The MDA is a reactive aldehyde, the major reactive electrophilic species known to elicit stress of toxic nature in cells and known to form covalent protein adducts which are referred to as advanced lipoxidation end products (ALE), found to be akin to advanced glycation end-products (AGE). SOD catalyses superoxide anions which are important reactive oxygen species in cells and involved in cell membrane damage. The elevation of GSH and SOD activities may be endogenous compensatory mechanism for prolonged over production of free radicals and oxidative stress. CAT is also an antioxidant enzyme located in peroxisomes and decomposes $H_2O_2$ to $H_2O$ and $O_2$. All these defensive antioxidant enzymes work in conjunction with each other, and thus, are able to protect from free radicals mediated oxidative injury/damage.
Treatment of diabetic (HFD + STZ) rats with rutin significantly improved the endogenous anti-oxidant enzymes (GSH, SOD and CAT) which might have enabled to prevent membrane damage by reducing lipid peroxidation process.

The hyper-triglyceridemia observed with diabetic (HFD + STZ) rats may be due to an increased absorption and formation of triglycerides in the form of chylomicrons following exogenous consumption of rich fat diet or through increased endogenous production of TG-enriched hepatic very low density lipoprotein (VLDL) and decreased TG -uptake in peripheral tissue. Hypercholesterolemia may also be due to increased dietary cholesterol absorption from small intestine following increased intake of high fat diet in diabetic condition. In the present study, there was a significant decrease in TC, TG, LDL, VLDL and also increase in HDL levels with the treatment of rutin (50 and 100 mg/kg).

The pharmacological and biochemical effects observed in diabetic (HFD+STZ) rats following rutin treatment are in conformity with the histoarchitecture alterations of β-islets of pancreas. In addition, treatment of diabetic rats with rutin and pioglitazone reduced hypertrophy of hepatocytes and lymphocytes infiltration.

In conclusion, it is stated that rutin treatment showed anti-diabetic activity in rat diabetic model by affecting many cellular events participating in the etiology of type 2 DM. Hence it may be a useful diabetic modulator and can be used as an adjuvant along with clinically effective anti-diabetic agents in the treatment of type 2 DM.

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