Decrease in serum matrix metalloproteinase-9 and matrix metalloproteinase-3 levels in Zucker fa/fa obese rats after treatment with swertiamarin

HB Vaidya PhD[1], S Giri MVSc[2], M Jain PhD[2], RK Goyal PhD[1]

HB Vaidya, S Giri, RK Goyal. Decrease in serum matrix metalloproteinase-9 and matrix metalloproteinase-3 levels in Zucker fa/fa obese rats after treatment with swertiamarin. Exp Clin Cardiol 2012;17(1):12-16.

Diabetes mellitus encompasses a group of chronic metabolic conditions associated with cardiovascular complications such as atherosclerosis, cardiomyopathy and nephropathy. In the present study, the authors investigated the beneficial effects of swertiamarin in diabetes and its associated cardiovascular complications in Zucker fa/fa rats. Six male Zucker fa/fa rats in each group were treated for 28 days with swertiamarin (75 mg/kg/day, intraperitoneally) or pioglitazone (30 mg/kg orally). Blood samples were collected and evaluated for several parameters. Elevated serum glucose, triglyceride, nonesterified free-fatty acid and cholesterol levels were found in untreated Zucker fa/fa rats. Serum matrix metalloproteinase (MMP)-9 and MMP-3 levels were also found to be significantly higher in untreated Zucker fa/fa rats. Treatment with swertiamarin significantly ($P<0.05$) reduced serum glucose, triglyceride, nonesterified free-fatty acid and cholesterol levels, and also reduced serum MMP-9 and MMP-3 levels compared with untreated rats. Swertiamarin also significantly ($P<0.05$) decreased serum levels of urea compared with untreated Zucker fa/fa rats. Overall, the data suggest that swertiamarin produced beneficial effects with respect to diabetes-induced cardiovascular complications such as atherosclerosis and nephropathy. A swertiamarin-induced decrease in serum MMP-9 and MMP-3 levels is one of the possible mechanisms responsible for improvement of these complications.

Key Words: *Atherosclerosis; Diabetes; Matrix metalloproteinase; Swertiamarin*

Cardiovascular complications are the leading cause of morbidity and mortality in patients with type 2 diabetes (1,2). As reported previously, macrovascular disease in diabetes is characterized by deposition of extracellular matrix in arteriolar basement membranes, which leads to alterations in the collagen-to-elastin ratio (3,4). These changes result in progressive stiffening of the large arteries, an increase in cardiac afterload, left ventricular mass and compromised coronary blood flow.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that degrade extracellular matrix proteins such as collagen and elastin. The activity of MMPs is influenced by the presence of tissue inhibitors of metalloproteinase (5). MMPs and their inhibitors are essential for cellular migration and vascular complications including unstable angina, plaque rupture and development of abdominal aortic aneurysms (6). The clinical significance of MMP-9, MMP-3 and MMP-2 has been reported in diabetes and diabetic complications such as atherosclerosis, obesity and nephropathy (7,8). Induction of MMPs in human arterial vasculature is downregulated in diabetic patients (9). Increases in plasma MMP-9 activity in the left ventricle of alloxan-induced diabetic wild-type mice has been previously documented (10). In human subjects, circulating MMP-9 levels are increased in type 2 diabetic patients with coronary artery disease, and elevated serum MMP-9 concentrations are linked to premature coronary atherosclerosis (11). It has also been proposed that an increase in plasma levels of MMP-9 occurs before the development of renal microvascular complications in patients with type 2 diabetes mellitus (12).

Swertiamarin is a secoiridoid glycoside, and data from our laboratory suggests that it is effective in diabetic rats and prevents diabetes-induced dyslipidemia, cardiomyopathy and nephropathy in various other animal models (13-15). The Zucker fa/fa rat is a valuable transgenic animal model to study diabetes and insulin resistance because the animals demonstrate early-onset, hyperplastic-hypertrophic obesity (16-21). In the present study, we investigated the beneficial effects of swertiamarin in diabetes and its cardiovascular complications in Zucker fa/fa rats.

METHODS

Isolation and characterization of swertiamarin

Swertiamarin was isolated and characterized according to previously described methods (22). Further confirmation was performed by recording melting point, infrared, nuclear magnetic resonance and mass spectrometry data, and comparing the results with published data and thin-layer chromatography using a standard sample of swertiamarin (22). Sample purity was verified by separation on high-performance thin layer chromatography using the following solvent system: ethyl acetate:methanol:water (0.7:0.2:0.1), and was found to be 98% pure. The standard swertiamarin sample was a gift from Professor Fumihiko Yoshizaki, Tohoku Pharmaceutical University, Sendai, Japan.

Animal experiments and treatment protocols

All procedures were performed in accordance with the Institutional Animal Care Committee as per the directions of the committee for the purpose of Control and Supervision of Experiments on Animals, under the Ministry of Animal Welfare Division, Government of India, New Delhi, India. Eighteen Zucker fa/fa rats weighing between 400 g and 600 g were used in the study. Three animals were housed per stainless steel cage in a room with controlled temperature (24°C) and lighting (alternating 12 h periods of light and dark). The animals were allowed access to water and food ad libitum. Zucker fa/fa rats were divided into three groups of six rats per group and treated for 28 days as follows: Zucker fa/fa rats vehicle control (NC); Pio-30 (pioglitazone 30 mg/kg orally); and swertiamarin (75 mg/kg intraperitoneally).

Food intake and body weight of the animals were recorded daily. At the end of the study, cumulative food intake and percentage change in body weight was calculated and reported.

Blood collection and biochemical analysis

On day 29, the animals were fasted for 18 h and blood samples were collected from the retro-orbital plexuses under normal ether anesthesia. The samples were centrifuged at 3000 rpm for 20 min at 4°C, the serum was separated and used for further analysis.

[1]*SPP School of Pharmacy and Technology Management, Mumbai;* [2]*Zydus Research Centre, Morriya, Ahmedabad, Gujarat, India*
Correspondence: Dr RK Goyal, SPP School of Pharmacy and Technology Management, NMIMS University, Ville Parle (West), Mumbai 400056, India. Telephone 91-224-233-2016, fax 91-222-618-5422, e-mail goyalrk@gmail.com

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TABLE 1
Effect of swertiamarin treatment on general parameters in obese Zucker fa/fa rats

	Normal control	Pio-30	Swertiamarin
Change in body weight, %	18.5±2.4	21.6±2.5*	18±2.2
Cumulative food intake, kg	3.42±1.8	4.62±1.1*	4.02±0.8*

*Data presented as mean ± SEM, number of animals in each group = 6. *Indicate significant difference compared with untreated Zucker fa/fa rats (P<0.05). Pio-30 Pioglitazone control (30 mg/kg orally); Swertiamarin Swertiamarin control (75 mg/kg intraperitoneally)*

Fasting blood glucose levels were measured using the GOD-POD glucose estimation kit (Span Diagnostics Pvt Ltd, India). The oral glucose tolerance test was performed on the second last day of the study after overnight fasting. Glucose solution at a dose of 3 mg/kg/5 mL water was administered orally to overnight fasted rats. Blood was collected from the retro-orbital sinus immediately after light ether anesthetization (0 min), and then at 30 min, 60 min, 90 min and 120 min after the oral glucose load and analyzed for plasma glucose.

Serum lipid content for all groups of animals was measured using commercially available kits (Span diagnostic Kit, India) including total cholesterol, triglycerides (TG) and nonesterified free fatty acid (NEFA). Serum creatinine and urea were estimated using a previously reported picrate method (23) and a urease method (24), respectively.

ELISA for MMP-9 and MMP-3

Serum concentrations of MMP-3 and MMP-9 were measured in duplicate using a commercially available ELISA kit (R&D Systems Inc, USA) according to the manufacturer's protocol. Briefly, 100 µL of MMP-3 or MMP-9 assay diluents were added to each well of a microplate followed by the addition of 50 µL of standard or control samples. After incubation for 2 h at room temperature on a constant shaker (500±50 rpm), the reaction solution was aspirated and the wells were washed four times with wash buffer; 200 µL of MMP-3 or MMP-9 conjugate was then added to each well and incubated for an additional 2 h on the shaker at room temperature. The aspiration/wash steps were then repeated as described above, followed by adding 200 µL of substrate solution to each well. The microplate was allowed to stand for 30 min at room temperature in the dark. After adding 50 µL of stop solution, the optical density at 450 nm of each well was determined. The MMP-3 and MMP-9 concentrations for each sample were calculated from the standard curve. The MMP-3 assay recognized both pro- and active forms of MMP-3. Intra-assay and interassay coefficients of variation were 4.8% and 7.7%, respectively. The minimum detectable dose of the MMP-3 assay was 0.16 ng/mL. The MMP-9 assay recognized both pro- and active forms of MMP-9; the intra-assay and interassay interassay coefficients of variation were 2.3% and 7.5%, respectively.

Statistical analysis

Values are presented as mean ± SEM. Statistical significance was calculated using one-way ANOVA followed by Tukey's multiple comparison tests to determine the level of significance. P<0.05 was considered to be statistically significant. The statistical analysis was performed using Graph Pad INSTAT version 3.0 (GraphPad, USA).

RESULTS

The effect of swertiamarin on food intake and body weight of Zucker fa/fa rats was assessed. At day 28 of the study, cumulative food intake was found to be significantly increased in swertiamarin- and pioglitazone-treated groups compared with the untreated Zucker fa/fa rats. Moreover, there was an increase in body weight in rats in the pioglitazone-treated group, but no change was observed in the swertiamarin-treated group (Table 1).

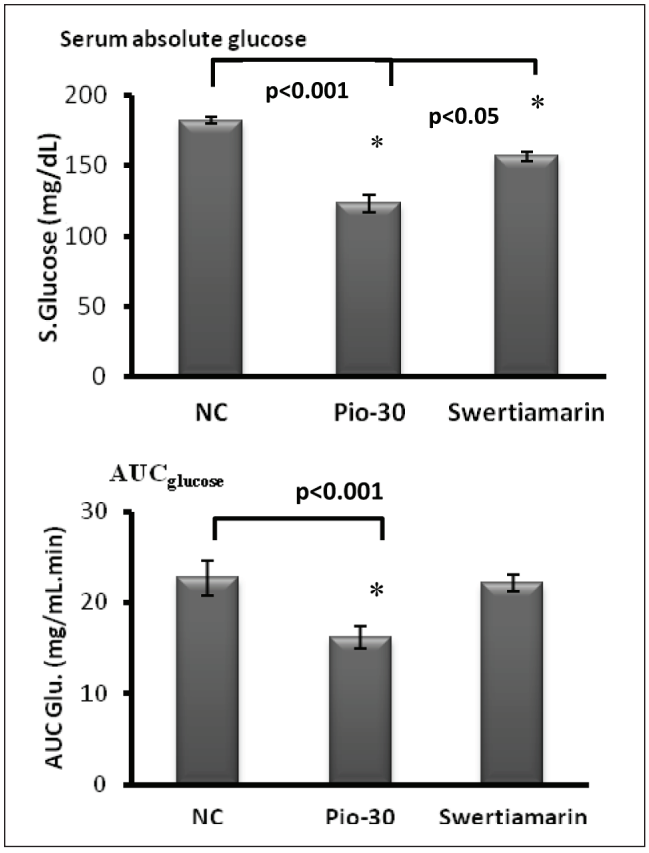

Figure 1) *Effect of swertiamarin on nonfasting glucose and glucose tolerance test of obese Zucker fa/fa rats. Each bar represents mean ± SEM, number of animals in each group = 6. AUC Area under the curve; Glu Glucose; NC Normal control; Pio-30 Pioglitazone control (30 mg/kg orally); S Serum; Swertiamarin Swertiamarian control (75 mg/kg intraperitoneally) once daily. *Statistically significant difference compared with NC Zucker fa/fa rats*

Effect of swertiamarin on serum glucose level and oral glucose tolerance test

Untreated Zucker fa/fa rats had significantly higher serum glucose levels and area under the curve $(AUC)_{glucose}$ level. Treatment with pioglitazone significantly (P<0.001) reduced glucose as well as $AUC_{glucose}$; treatment with swertiamarin also produced a significantly (P<0.05) reduced glucose level compared with untreated Zucker fa/fa rats (Figure 1), but not $AUC_{glucose}$.

Estimation of lipid profile from blood samples

Higher serum cholesterol, TG and NEFA levels in untreated Zucker fa/fa rats. Treatment with swertiamarin and pioglitazone signifiantly reduced serum cholesterol, TG and NEFA levels (Figure 2).

Effect of swertiamarin on serum MMP-9 and MMP-3 levels

MMP-3 and MMP-9 levels were found to be significantly higher in untreated Zucker fa/fa rats than in the treated groups (Figure 3). Treatment with swertiamarin significantly decreased the activity of MMP-9 and MMP-3. The decrease in MMP-9 activity was greater than the decrease in MMP-3 activity. Treatment with pioglitazone also caused significant reduction in the levels of both proteins.

Effect of swertiamarin on kidney parameters

Untreated Zucker fa/fa rats were found to have high serum urea and creatinine levels. Treatment with pioglitazone induced a significant (P<0.05) increase in the level of serum urea; however, there was no significant change in serum creatinine level compared with the untreated Zucker rats. Treatment with swertiamarin also produced

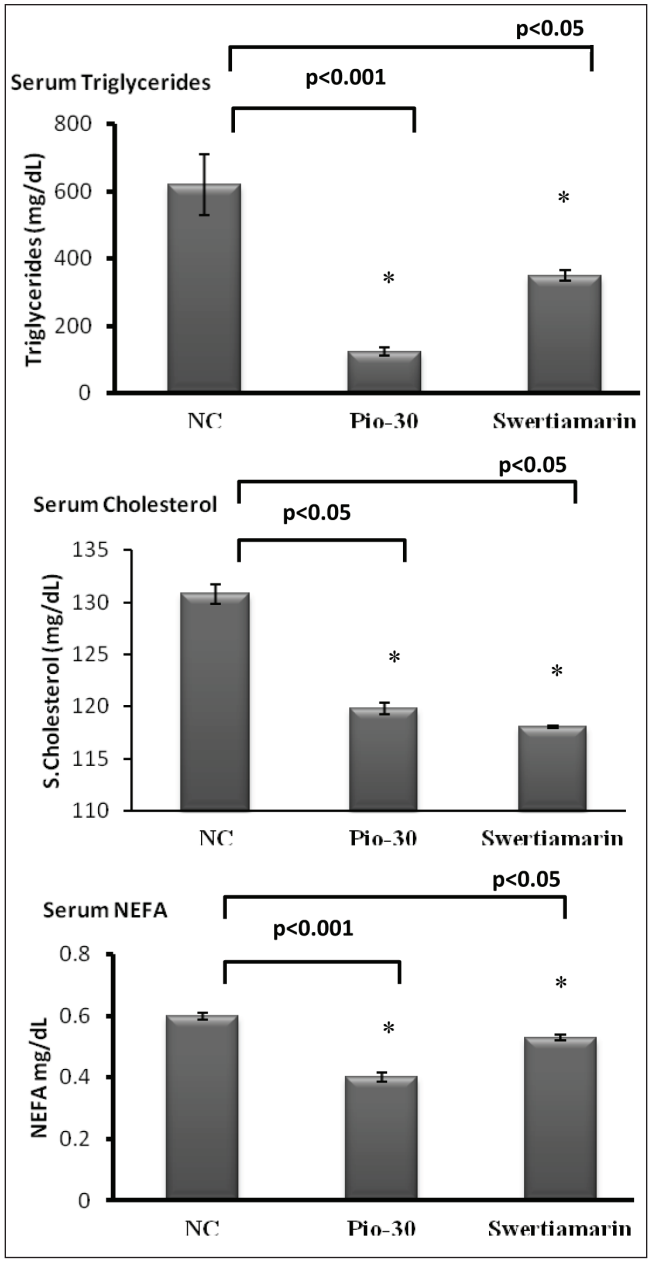

Figure 2) *Effect of swertiamarin treatment on serum cholesterol, triglyceride and nonesterified fatty acid (NEFA) levels. Each bar represents mean ± SEM. NC Normal control; Pio-30 Pioglitazone treated (30 mg/kg orally); S Serum; Swertiamarin Swertiamarin treated (75 mg/kg intraperitoneally). *Statistically significant difference compared with NC Zucker fa/fa rats*

significant reduction in serum levels of urea compared with the untreated Zucker fa/fa rats (Table 2); however, no significant change was observed in creatinine level.

DISCUSSION

The present investigation was planned due to results of an earlier report from our laboratory that suggested that swertiamarin produced significant effects, not only on serum glucose and insulin levels, but also reduced serum cholesterol and triglyceride levels in streptozotocin-induced type 2 diabetes (In press). Swertiamarin was also reported to produce a statin-like effect in high cholesterol diet-induced hyperlipidemia in rats (14). To date, there have been no reports of swertiamarin producing a decrease in serum matrix MMP-9 and MMP-3 levels, and with significant reduction in serum glucose, TG, cholesterol and NEFA levels.

TABLE 2
Effect of swertiamarin on kidney function test of obese Zucker fa/fa rats

	Normal control	Pio-30	Swertiamarin
Serum urea, mg/dL	37.7±3.1	55.2±1.2	37.3±2.9*
Serum creatinine, mg/dL	0.51±0.02	0.54±0.05	0.45±0.02

*Data presented as mean ± SEM. Number of animals in each group = 6. Pio-30 Pioglitazone control (30 mg/kg orally); Swertiamarin Swertiamarin control (75 mg/kg intraperitoneally). *Statisitically significant difference compared with untreated Zucker fa/fa rats. (P<0.05)*

Swertiamarin treatment was associated with a significant increase in food intake; however, there was no significant gain in weight observed compared with the untreated Zucker fa/fa rats. Moreover, the pioglitazone-treated group demonstrated a significant increase in weight compared with untreated Zucker fa/fa rats.

Untreated Zucker fa/fa rats were found to be hyperinsulinemic and obese, in addition to having a significantly higher nonfasting glucose and $AUC_{glucose}$ level. Treatment with pioglitazone significantly (P<0.001) reduced nonfasting glucose and $AUC_{glucose}$ levels compared with the untreated Zucker fa/fa rats. Treatment with swertiamarin also produced a significant (P<0.05) decrease in nonfasting glucose level compared with untreated Zucker rats; however, swertiamarin treatment was not assocuiated with a significant reduction in $AUC_{glucose}$ after 28 days of treatment.

Insulin resistance may be responsible for a dyslipidemic condition because in normal circumstances, insulin activates the enzyme lipoprotein lipase, which hydrolyzes TG; thus, insulin deficiency results in failure to activate lipoprotein lipase, resulting in dyslipidemia. Acute insulin deficiency initially causes an increase in free fatty acid mobilization from adipose tissue (25), and with longer insulin deficiency, the liver converts free fatty acids into ketone bodies and very-low-density lipoprotein triglyceride secretion diminishes (26). At the same time, lipoprotein lipase activity declines, resulting in impaired clearance of low-density lipoprotein and chylomichons from plasma. Thus, insulin resistance in patients leads to compensatory hyperinsulinemia, which is associated with increased low-density lipoprotein and reduced high-density lipoprotein concentrations (27). Zucker fa/fa rats are a transgenic model for insulin resistance and obesity. Untreated Zucker fa/fa rats were found to be hyperinsulinemic and hyperlipidemic, with elevated TG, NEFA and cholesterol levels. Treatment with swertiamarin (75 mg/kg, intraperitoneally) caused a significant decline in serum cholesterol, TG and NEFA levels compared with untreated Zucker fa/fa rats. The same effects were also produced in the pioglitazone-treated group (30 mg/kg orally).

MMPs play a major role in the pathogenesis of atherosclerosis and restenosis after angioplasty or vein graft stenosis. As previously reported, the extracellular matrix is remodelled by MMPs (28). The involvement of MMPs in the pathogenesis of diabetes (and in diabetic complications) has also been well documented. In diabetes, downregulation of MMP induction and activation systems occurs in human arterial vasculature (9). In human subjects, circulating MMP-9 levels are increased in type 2 diabetic patients with coronary artery disease, and elevated serum MMP-9 concentrations have been linked to premature coronary atherosclerosis (11). It has also been proposed that increases in serum levels of MMP-9 occur before the development of renal microvascular complications in patients with type 2 diabetes mellitus (12). Recent reports suggest that statins reduce MMP-9 secretion by macrophages (29) and also lower levels of MMP-3 and MMP-9 in established abdominal aortic aneurysms (30). In the present study, we found that swertiamarin treatment significantly decreased not only serum TG (P<0.05) and cholesterol (P<0.05) levels compared with the untreated Zucker fa/fa rats (Figure 2) but also produced significant increases in food intake. Swertiamarin treatment, however, did not lead to significant weight gain compared with the untreated Zucker fa/fa rats.

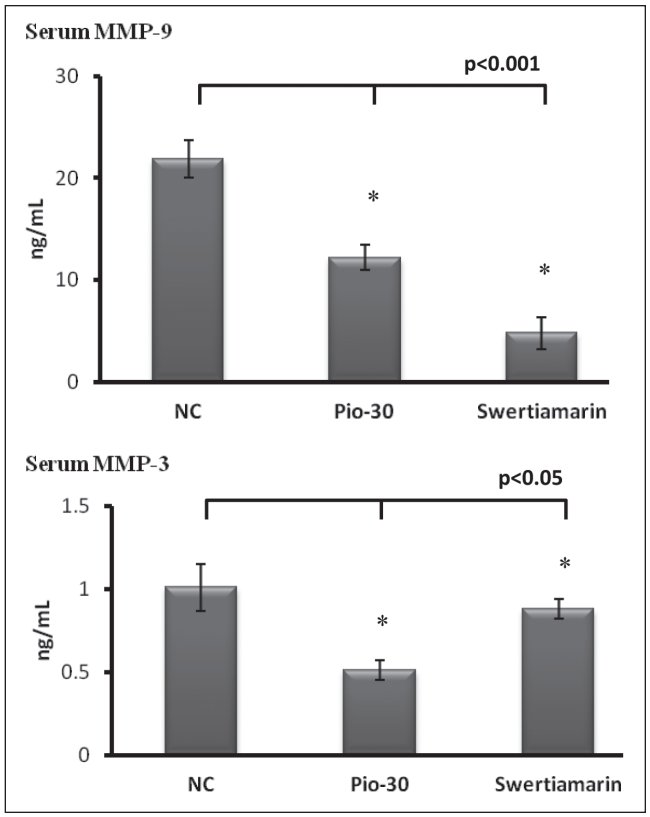

Figure 3) *Effect of swertiamarin treatment on serum matrix metalloproteinase (MMP)-9 and MMP-3 levels. Each bar represents mean ± SEM. Number of animals in each group = 6. NC Normal control; Pio-30 Pioglitazone treated (30 mg/kg orally); Swertiamarin Swertiamarin treated (75 mg/kg intraperitoneally). *Statistically significant difference compared with NC Zucker fa/fa rats*

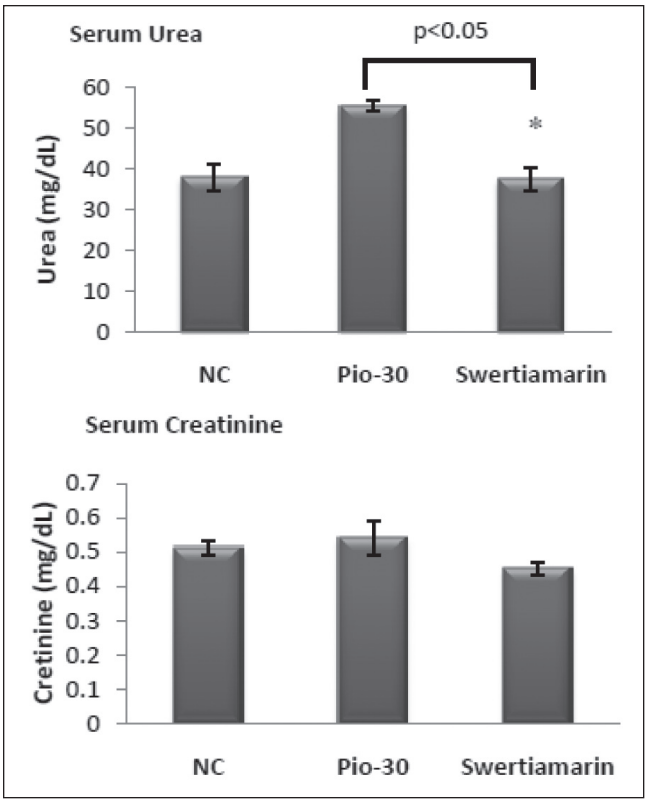

Figure 4) *Effect of swertiamarin treatment on serum urea and creatinine levels. Each bar represents mean ± SEM. Number of animals in each group = 6. NC Normal control; Pio-30 Pioglitazone treated (30 mg/kg orally); Swertiamarin Swertiamarin treated (75 mg/kg intraperitoneally). *Statistically significant difference compared with NC Zucker fa/fa rats*

In the present study, we demonstrated that swertiamarin, a natural HMG Co-A reductase inhibitor, significantly decreased not only serum MMP-3 (P<0.05) and MMP-9 (P<0.001) levels but also serum TG, NEFA and total cholesterol in Zucker fa/fa rats compared with the untreated Zucker fa/fa rats, and that these effects were similar to that of the pioglitazone-treated group. Pioglitazone significantly (P<0.05) reduced MMP-3 and MMP-9 levels (Figure 3) compared with the untreated Zucker rats; however, in the swertiamarin-treated group, the effect on MMP-9 was more significant compared with MMP-3. These data are consistent with those reported in a previous publication in which pioglitazone treatment resulted in a decrease in serum MMP-9 levels in type 2 diabetic animals (31).

Serum creatinine and urea are biomarkers of kidney function, which are found to be significantly increased in patients with diabetes or other kidney disorders. Serum urea levels were significantly increased in untreated animals (Figure 4); however, treatment with swertiamarin significantly (P<0.05) lowered serum urea levels compared with untreated animals (Table 2); however, no significant changes in creatinine level were observed.

Overall, the data suggest that the elevated levels of MMP-9 and MMP-3 in Zucker fa/fa rats were significantly reduced by the treatment with swertiamarin and pioglitazone independently. Moreover, treatment with swertiamarin significantly reduced serum urea levels compared with the untreated Zucker fa/fa rats. Thus, we conclude that swertiamarin produces beneficial effects in diabetes-induced cardiovascular complications such as atherosclerosis and nephropathy. A swertiamarin-induced decrease in serum MMP-9 and MMP-3 levels is one of the possible mechanisms responsible for improvement of these complications.

ACKNOWLEDGEMENTS: The authors thank the Department of Science and Technology, New Delhi for the research grant, and the Zydus-Reseach Centre, Ahmedabad for providing facilities and infrastructure.

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