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SILDENAFIL ALLEVIATES INSULIN SENSITIVITY VIA ATTENUATING OXIDATIVE STRESS AND PROINFLAMMATORY CYTOKINE PRODUCTION IN DIABETIC RATS

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ABSTRACT

The present study was hypothesized to assess the effect of sildenafil, a phosphodiesterase inhibitor, on proinflammatory cytokines and hyperglycemia-mediated oxidative stress in type 2 diabetic rats. Diabetes was induced by feeding rats with high fat diet for 2 weeks followed by an intraperitoneal injection of streptozotocin. In the diabetic control group, levels of glucose were significantly increased, while serum insulin level was decreased. In addition, serum TNF-α, IL-6, IL-1β, total cholesterol, triglycerides, LDL-cholesterol and vLDL-cholesterol were significantly elevated in diabetic rats. Moreover, hepatic MDA was significantly increased in diabetic rats, while GSH content as well as GPx and SOD activities were significantly decreased. Both doses of sildenafil alleviated the altered parameters. Collectively, sildenafil exerts protection to type 2 diabetic rats by alleviating insulin sensitivity, potentiating the antioxidant defense system and suppressing proinflammatory cytokine production.

KEYWORDS: **Sildenafil, oxidative stress, diabetes mellitus, proinflammatory cytokines.**

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INTRODUCTION

Diabetic mellitus (DM) is one of the most important health problems in the world, especially in developing countries[1]. It is a group of metabolic diseases characterized by hyperglycemia resulting from the defects in insulin secretion, insulin action, or both[2,3]. The chronic hyperglycemic condition in diabetes is associated with long term damage, dysfunction, and failure of various organs, such as eyes, kidneys, nerves, heart and blood vessels[2,4]. Acute inflammation is utilized by the immune system to effectively isolate and eliminate pathogenic microorganisms. Among the most commonly observed cytokines in inflammatory microenvironments is tumor necrosis factor (TNF)-α[5]. This cytokine is produced by a variety of immune cells including macrophages and lymphocytes[6] and is pleiotropic in nature[7]. TNF-α is also produced by adipose tissue[8,9] and it is thought to play a major role in the Metabolic Syndrome (MS), which is characterized by insulin resistance and inflammation[9]. TNF-α can elicit an insulin-resistant state, characterized by an impaired ability of insulin to suppress hepatic glucose production and to stimulate peripheral glucose uptake[10]. Also, TNF-α is implicated to increase the circulating level of free fatty acids and thus indirectly contributes to the pathogenesis of insulin resistance[11]. Similarly, increases in the proinflammatory cytokine, IL-6, lead to a reduction in insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation, a decreased association between the PI-3 kinase and IRS-1 and an inhibition of insulin-dependent activation of Akt[12,13]. In addition, IL-6 has also been found to reduce lipoprotein lipase (LPL) activity in the adipose tissue of mice and in 3 T3-L1 adipocytes *in vitro*[14]. Sildenafil is a drug commonly used in the treatment of erectile dysfunction. It inhibits the metabolism of cyclic guanosine monophosphate (cGMP), resulting in increased relaxation of the smooth muscle surrounding arterioles supplying the human corpus cavernosum[15]. Recently, we had addressed the beneficial effects of sildenefil citrate in acetic acid-induced colitis in rats[16]. Thus, the current study was designed to investigate the ameliorative potential of sildenafil citrate on proinflammatory cytokines and hyperglycemia-mediated oxidative stress in type 2 diabetic rats.

MATERIALS AND METHODS

(i) Chemicals

Streptozotocin (STZ) was purchased from Sigma Chemicals Co., St. Louis, MO, USA. Sildenafil citrate was supplied from Pfizer Inc. (Pfizer, Egypt), stored at 2-4 °C and protected from sunlight. All other chemicals were of analytical grade and were obtained from standard commercial supplies.

(ii) Experimental animals

Male albino rats weighing about 160-180 g were used as experimental animals in the present investigation. The animals were housed in standard polypropylene cages with stainless steel good aerated covers and maintained under controlled room temperature (22±2 ◦C) with 12 hrs light - dark cycle and were fed a standard diet of known composition, and water ad libitum. The animals used in the present study were maintained in accordance with the principles and guidelines of the Canadian Council on Animal Care as outlined in “Guide for the Care and Use of Laboratory Animals”[17].

(iii) Induction of diabetes mellitu

Type 2 DM was experimentally induced by feeding a high fat diet (HFD) for an initial period of 2 weeks followed by an interapritoneal injection of 35 mg/kg b. wt streptozotocin dissolved in citrate buffer pH 4.5[18]. The composition and preparation of HFD were described elsewhere[19]. Seven days after the injection, rats were screened for serum glucose levels. Rats having serum glucose ≥ 200 mg/dl, after 2 hours of glucose intake, were considered diabetic..

(iv) Experimental design

The experimental animals were divided into four groups, each group comprising six rats designated as follows: group 1 served as normal control rats; group 2 served as diabetic control rats; group 3 served as diabetic rats

administered 5 mg/kg b.wt sildenafil orally for 4 weeks; and group 4 served as diabetic rats administered 10 mg/kg b.wt sildenafil orally for 4 weeks. The dosage was adjusted every week according to any change in body weight to maintain similar dose per kg body weight of rat over the entire period of study for each group. By the end of the experiment, animals were sacrificed and blood samples and liver were obtained.

(v) Biochemical studies

The level of serum glucose was estimated spectrophotometrically according to the method of Trinder [20], using commercial diagnostic kit (Spinreact, Spain). Serum insulin and adiponectin levels were determined using specific ELISA kit (R&D Systems, USA), according to the manufacturer instructions. Insulin resistance was evaluated by HOMA-IR[21] as follows:

HOMA-IR=Fasting insulin (μU/ml) x Fasting glucose (mmol/L)/22.5. Serum total cholesterol[22], triglycerides[23] and HDL-cholesterol[24] were assayed using commercial diagnostic kits (Spinreact, Spain). Serum vLDL-cholesterol concentration was calculated according to Nobert[25] formula (vLDL-cholesterol=triglycerides/5). Serum LDL-cholesterol level was calculated from Friedewald [26] formula (LDL-cholesterol = total cholesterol – triglycerides/5 – HDL-cholesterol). Serum levels of the proinflammatory cytokines, TNF- α, IL-6 and IL-1β, were determined by specific ELISA kits according to the manufacturer's instructions (R&D Systems, USA). The concentration of proinflammatory cytokines was determined spectrophotometrically at 450 nm. Standard plots were constructed by using standard cytokines and the concentrations for unknown samples were calculated from the standard plot. Lipid peroxidation, reduced glutathione content, and superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were also measured in liver homogenate according to the methods of Preuss *et al* [27], Beutler *et al* [28], Marklund and Marklund[29] and Kar and Mishra[30], respectively.

(vi)Statistical analysis

The data were analyzed using the one-way analysis of variance (ANOVA) (PC-STAT, University of Georgia, 1985) followed by LSD test to compare various groups with each other. Results were expressed as mean ± SE and values of $P>0.05$ were considered non-significantly different, while those of $P<0.05$ and $P<0.01$ were considered significant and highly significant, respectively.

RESULTS

HFD followed by STZ produced a very highly significant elevation ($P<0.001$; LSD) of both fasting and postprandial glucose levels as compared with normal rats. Treatment of diabetic rats with both 5 and 10 mg/kg sildenafil produced a pronounced amelioration of the elevated serum blood glucose levels (Figure 1).

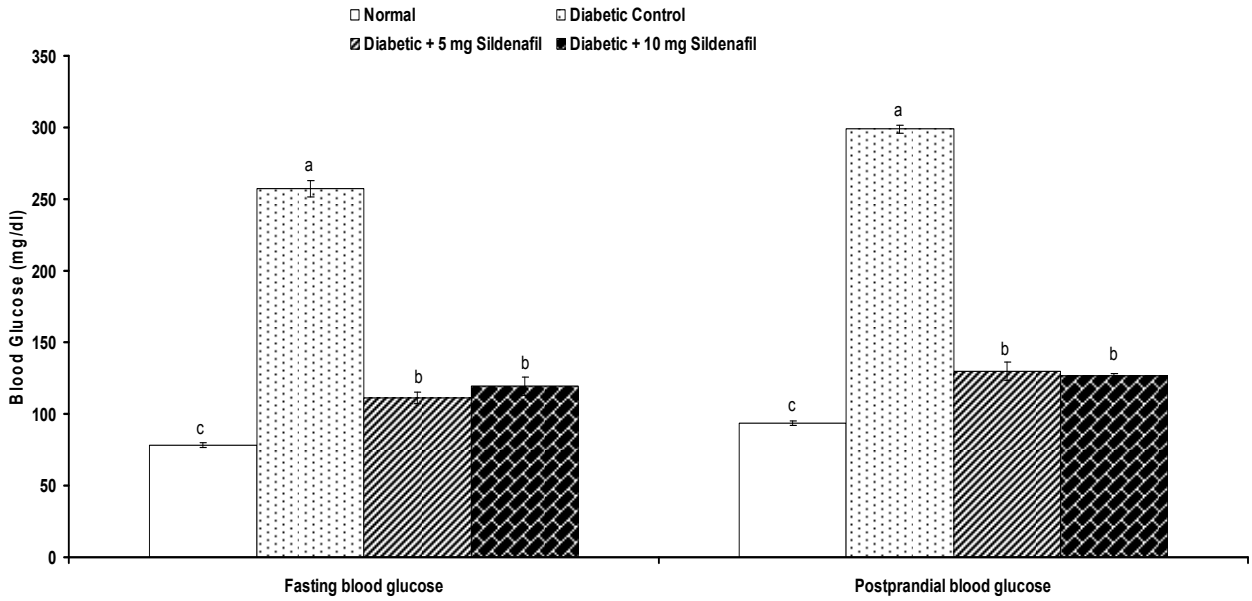

Figure 1
Effect of sildenafil on fasting and postprandial blood glucose levels of normal, diabetic control and diabetic treated rats.

The recorded values of fasted diabetic rats showed a highly significant decrease in serum insulin concentration as compared with the normal control rats. Treatment with both 5 and 10 mg/kg sildenafil produced an increase in serum insulin concentration (Figure 2).

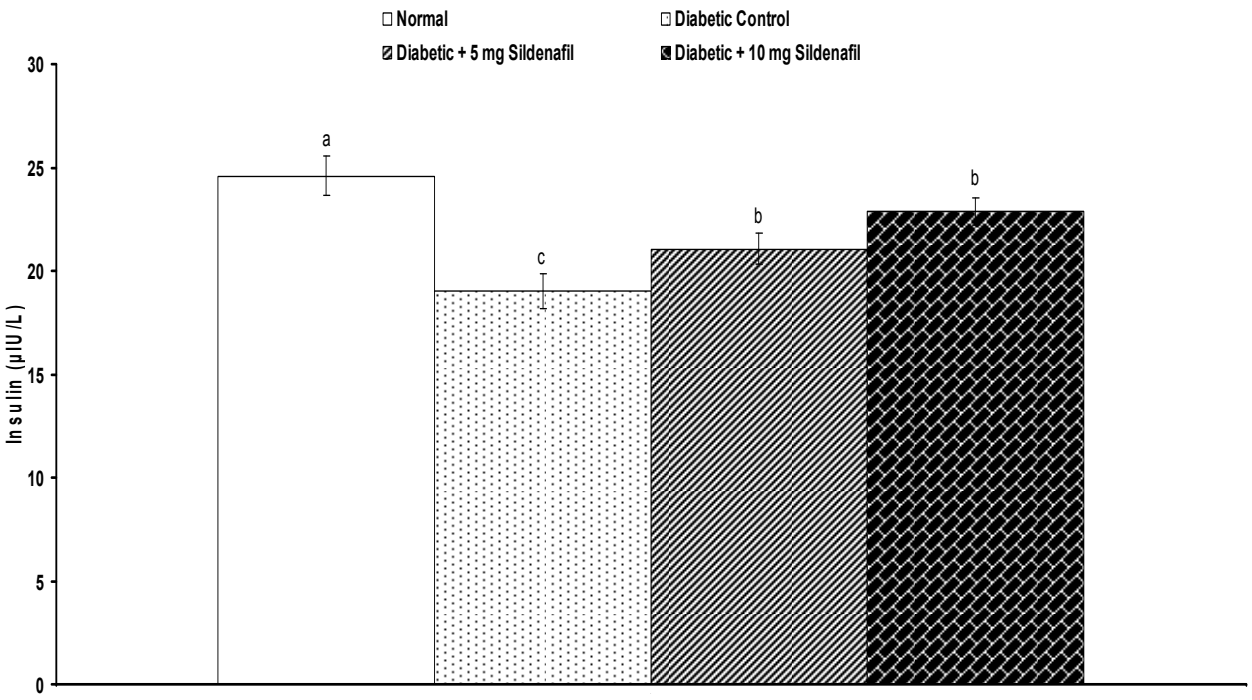

Figure 2
Effect of sildenafil on serum insulin concentration of normal, diabetic control and diabetic treated rats

Sildenafil at 10 mg dose appeared to be more effective in increasing serum insulin level than the 5 mg dose. On the other hand, diabetic rats showed a significant (p<0.01; LSD) elevation of HOMA-IR that was decreased significantly upon administration of either low or high dose of sildenafil as illustrated in Figure 3.

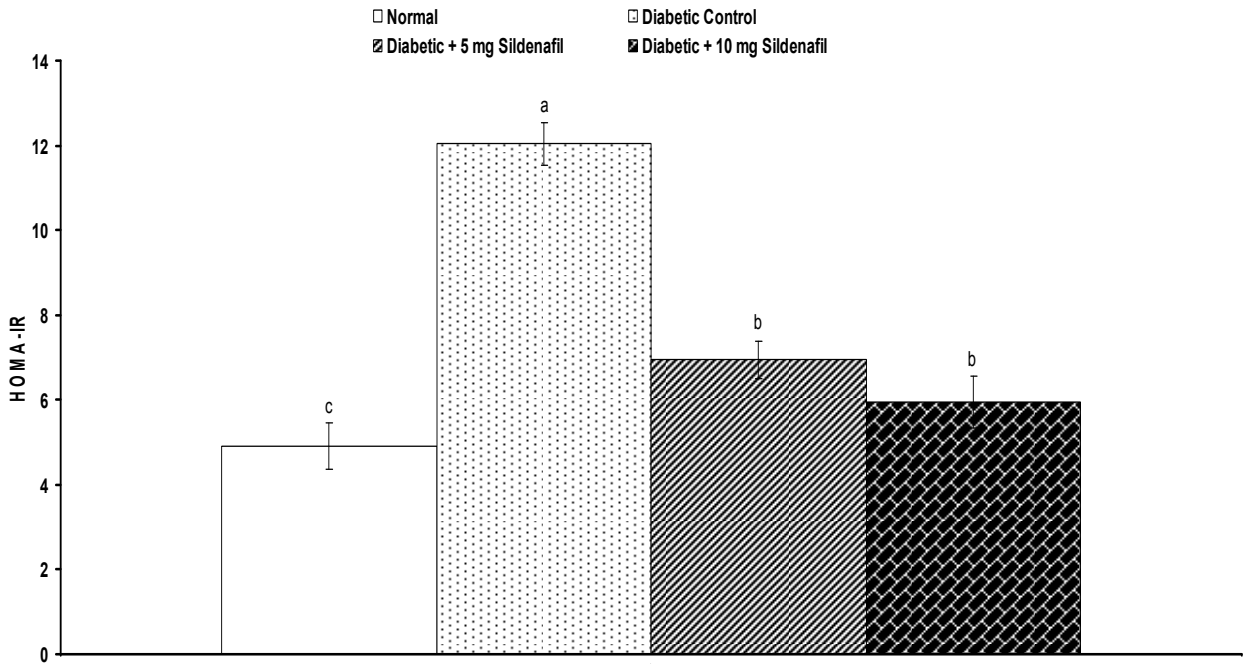

Figure 3
Effect of sildenafil on HOMA-IR of normal, diabetic control and diabetic treated rats

Data on the effect of sildenafil on lipid profile of diabetic rats were presented in Table 1.

Table 1
Lipid profile of normal, diabetic and diabetic rats treated with sildenafil

Parameter / Group	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	vLDL-cholesterol (mg/dl)
Normal	65.81 ± 2.21^{c}	51.10 ± 2.51^{c}	37.62 ± 1.87^{c}	20.07± 1.61^{c}	10.22 ± 0.50^{c}
Diabetic control	138.37 ± 7.16^{a}	133.24 ± 8.20^{a}	22.67 ± 1.89^{a}	89.12 ± 11.14^{a}	26.63 ± 1.64^{a}
Diabetic + 5 mg/kg Sildenafil	93.51 ± 2.29^{b}	98.79 ± 10.27^{b}	28.96 ± 2.22^{b}	43.13 ± 3.42^{b}	19.75 ± 2.06^{b}
Diabetic + 10 mg/kg Sildenafil	95.81 ± 2.67^{b}	96.63 ± 4.56^{b}	29.11 ± 2.13^{b}	49.45 ± 3.54^{b}	19.33 ± 0.90^{b}
F- prob	P< 0.001	P< 0.001	P< 0.001	P< 0.001	P< 0.001
LSD at 5%	12.17	14.73	4.24	12.80	2.94
LSD at 1%	16.59	20.09	5.78	17.46	4.02

- Data are expressed as Mean ± SE. Number of animals in each group is six.
- Means which share the same superscript symbol (s) are not significantly different.

Diabetic rats exhibited a highly significant increase (LSD; P<0.01) in serum total cholesterol, triglycerides, LDL- and vLDL-cholesterols as compared with the non-diabetic group. Moreover, HDL-cholesterol was affected in an opposite manner, as it was highly significant decreased (LSD; P<0.01) in diabetic rats. The administration of both tested doses led to marked amelioration of all parameters of the altered lipid profile. The effect of sildenafil on serum TNF-α, IL-6 and IL-1β of normal and diabetic rats was illustrated in figures (4).

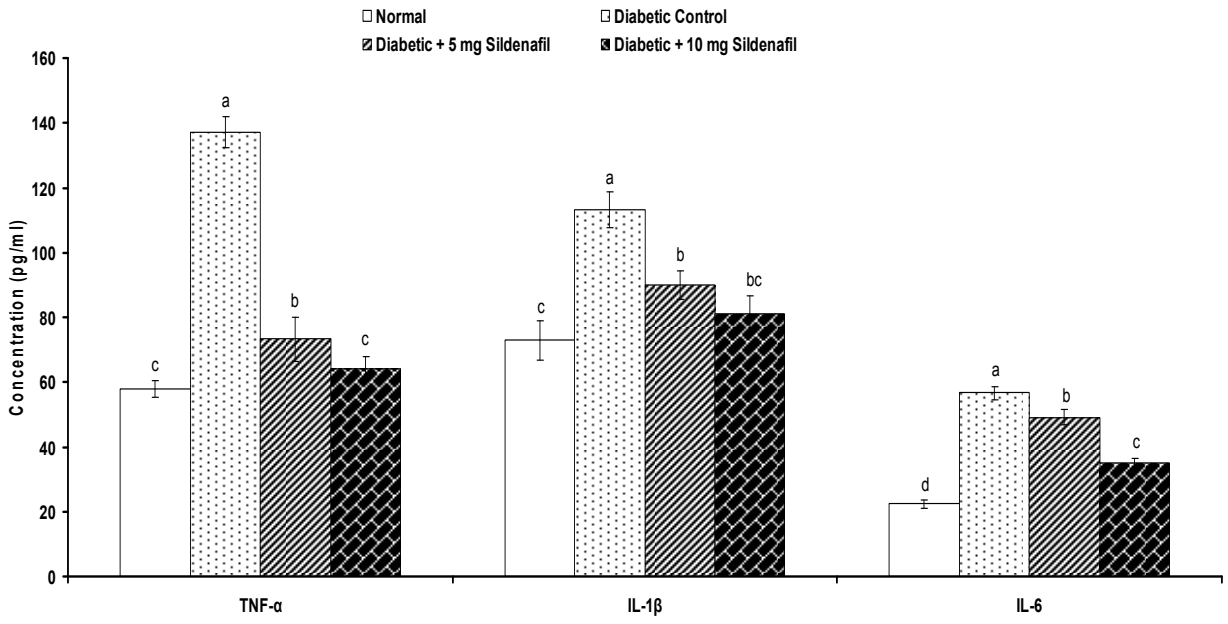

Figure 4
Serum TNF-α, IL-1β and IL-6 of normal, diabetic control and diabetic rats treated with sildenafil.

In diabetic rats, both doses of sildenafil induced a highly significant (P<0.01) decrease of TNF-α,IL-6 and IL-1β as compared to the control rats.

Table 2
Liver MDA, GSH, GPx and SOD of normal, diabetic and diabetic rats treated with sildenafil.

Group	Parameter	MDA (nmol/100 mg tissue)	GSH (nmol/100 mg tissue)	GPx (U/g tissue)	SOD (U/g tissue)
Normal		20.60 ± 1.36^{d}	60.59 ± 3.20^{a}	71.73 ± 1.49^{a}	61.71 ± 7.87^{a}
Diabetic control		41.17 ± 1.18^{a}	34.05 ± 1.62^{d}	41.25 ± 2.18^{d}	38.28 ± 3.67^{b}
Diabetic + 5 mg/kg Sildenafil		30.39 ± 0.76^{b}	42.73 ± 2.22^{c}	52.88 ± 3.28^{c}	48.64 ± 8.14^{ab}
Diabetic + 10 mg/kg Sildenafil		26.36 ± 1.01^{c}	54.33 ± 3.29^{b}	63.92 ± 3.54^{b}	58.59 ± 7.58^{a}
F- prob		P< 0.001	P< 0.001	P< 0.001	P< 0.01
LSD at 5%		2.29	5.58	5.73	14.71
LSD at 1%		3.12	7.60	7.81	20.06

- Data are expressed as Mean ± SE. Number of animals in each group is six.
- Means which share the same superscript symbol (s) are not significantly different.

Table 2 illustrates the effect of sildenafil administration on the levels of liver lipid peroxides and antioxidant markers of diabetic rats. The elevated level of lipid peroxides observed in liver of diabetic rats was potentially (p<0.01; LSD) improved by the treatment of diabetic groups of rats with both doses of sildenafil. Conversely, hepatic GSH content and GPx and SOD activities were significantly declined in HFD/STZ diabetic rats. Treatment of the diabetic rats with doses of sildenafil potentially increased (p<0.01; LSD) GSH as well as antioxidant enzymes activity, however the low dose of sildenafil non-significantly (P>0.05) affects the hepatic SOD activity.

DISCUSSION

HFD/STZ–induced diabetic rat is one of the animal models of human diabetes mellitus[19]. The rats fed with HFD can result in insulin-resistance mainly through Randle or glucose–fatty acid cycle[31]. It was also reported that in

addition to a direct effect on glucose, which is characteristic of this model, other pathophysiological changes were seen, such as insulin resistance in adipose tissue[19] and diabetic kidney lesions such as glomerulosclerosis and proteinuria[32]. Also, HFD feeding to rodents was shown to affect the respiratory capacity, reactive oxygen species (ROS) generation, fatty acid beta oxidation, mitochondrial ADP/ATP translocator inhibition and regulation of kinases involved in carbohydrate and lipid metabolism[33]. Since the combination of high fat diet-fed and low-dose streptozotocin treated rat which serves as an alternative animal model for diabetes has been proved to be suitable for testing antidiabetic agents[34], so this model was chosen to carry out our evaluations. In the present study, diabetic group rats exhibited significantly elevated fasting and postprandial blood glucose, and HOMA-IR, accompanied with diminished serum insulin levels as compared to normal rats. Hence, it is proposed that insulin resistance has been developed in these animals. Therefore, this rat model exhibits hyperglycemia with insulin resistance that would closely reflect the natural history and metabolic characteristics of diabetic humans, and it is further sensitive to pharmacological testing. Treatment of the diabetic rats with either 5 or 10 mg sildenafil citrate potentially improved the altered blood glucose. These results may be due amelioration of insulin sensitivity. The current study is in agreement with results of Ayala *et al* [35] who showed that chronic inhibition of phosphodiesterase-5 improves insulin action in a mouse model of diet-induced obesity and insulin resistance. One potential mechanism by which phosphodiesterase-5 inhibition may improve insulin action is prevention of endothelial dysfunction. Recent evidence supports the notion that endothelial dysfunction may be causative of insulin resistance and type 2 diabetes[36]. Endothelial dysfunction is characterized by a decrease in NO levels, reducing cGMP production and impairing muscle glucose uptake[37]. Thus, it is possible that preventing a decrease in cGMP levels by inhibiting phosphodiesterase-5 intervenes downstream of the site of endothelial dysfunction, resulting in improved insulin action on muscle glucose uptake. It is also possible that the enhanced insulin action in sildenafil-treated diabetic rats resulted from an effect on the central nervous system. Sildenafil has been shown to cross the blood-brain barrier, and phosphodiesterase-5 expression has been detected in the brain[38]. Thus, signaling through cGMP in the central nervous system may play a role in the regulation of insulin action and energy homeostasis as stated by Ayala *et al* [35]. Diabetic dyslipidemia has long been shown to have a strong relation with coronary heart disease [39,40], which is the most dangerous and life threatening complication of diabetes and the risk of coronary heart disease in diabetes increases two- or more folds[41]. The rise in blood glucose was accompanied with a marked increase in total cholesterol, LDL-cholesterol, triglycerides and reduction in HDL-cholesterol in HFD/STZ diabetic rats. The altered lipid and lipoprotein profiles were significantly reversed after 4 weeks of both 5 and 10 mg sildenafil supplementation to the diabetic rats. The significant control in the serum lipid levels in treated diabetic rats might have been due to the increase insulin sensitivity following sildenafil administration. An important finding of the present study was that sildenafil, in a dose dependent manner, attenuated production of the pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β, which are believed to play a significant role in the pathogenesis of DM. TNF-α affects intracellular insulin signaling in fat, skeletal muscle, endothelial cells, and other insulin-responsive tissues by inhibiting kinase activities in the insulin-signaling pathway[42]. TNF-α has been shown to increase plasma triglycerides and concentrations of very low density lipoproteins[43], as well as lipolysis in mouse, rat, and human fat cells[44]. TNF-α reduces insulin-stimulated receptor tyrosine kinase activity at low concentrations and can also decrease the expression of the insulin receptor IRS-1 and GLUT-4 at higher concentrations as well as increases the phosphorylation of serine 307 in IRS-1, thus impairing its ability to bind to the insulin receptor and initiate downstream signaling[42]. In addition, increases in IL-6 leads to a reduction in IRS-1 tyrosine phosphorylation, a

decreased association between the PI-3 kinase and IRS-1 and an inhibition of insulin-dependent activation of Akt[12,13]. Thus, the observed amelioration of insulin sensitivity may be mediated via attenuating proinflammatory cytokines by sildenafil.

Increased oxidative stress and impaired antioxidant defense mechanism are important factors in the pathogenesis and progression of DM and other oxidant-related diseases[3]. The potentially antioxidative effects of PDE-5 inhibition in diabetes[40] could be confirmed in the present study. The administration of both doses of sildenafil to diabetic group of rats significantly reverted back the altered levels of malondialdehyde and the antioxidants, GSH, GPx and SOD, which in turn reveal the antioxidant potential of sildenafil. There is evidence that biological responses triggered by oxidative products are associated with lipid peroxidation derivatives, which are able to induce various pathogenic intracellular signals involving calcium, G-proteins, cAMP, cGMP, phospholipase C and D, protein kinase C, ceramide, and MAP kinase cascade leading to cellular dysfunction[46]. Thus increasing cyclic nucleotides by use of PDE inhibitors could overcome to oxidative stress-induced cellular dysfunctions and apoptosis. Supporting this conclusion, Polte and Schroder[47] reported an antioxidant property for nitric oxide (NO) donors in vascular endothelium through concerted action of cGMP and cAMP. They showed that S-nitroso-N-acetyl-d,l-penicillamine (SNAP) protects from TNF-mediated endothelial cell toxicity. The cytoprotection by SNAP was completely abolished by the adenylyl cyclase inhibitor 2,5-dideoxyadenosine and mimicked by 8-bromo cAMP or forskolin. In conclusion, biochemical findings of the present study indicate that the PDE inhibitor sildenafil citrate exerts protection to HFD/STZ diabetic rats against hyperglycemia-mediated oxidative stress. This could be due to the prevention or inhibition of lipid peroxidation and alleviating the antioxidant system. In addition, the hypolipidemic and insulin sensitizing effects of sildenafil may be attributed to its anti-inflammatory effect.

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