Original Article

Therapeutic Implications of Thymoquinone in the Management of Diabetes mellitus and its Complications

Samir A.E. Bashandy*, Gehad A Abdel Jaleel, Heba M.I. Abdallah and Sahar E.S. Harraz

Department of Pharmacology, National Research Centre, Egypt

ABSTRACT

Background: Hyperglycemia leads to development of complications associated with diabetes via oxidative stress. Thymoquinone (TQ), derived from *Nigella sativa* seed, has an antioxidant activity. The present study investigated the attenuation of some diabetes complications by TQ in rats.

Main methods: Male albino rats were assigned to three groups of ten animals each: group I, control; group II, streptozotocin (STZ) group and group-III, diabetic rats treated with TQ (40mg/Kg bw) through gastric tube for three weeks. Blood and tissue samples were collected for measurement of oxidative stress biomarkers, inflammatory markers, lipid profile, blood cells count, kidney and liver function tests.

Results: Findings of this study showed that WBC (White blood cell) count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, total cholesterol (TC), triglycerides (TG), low density lipoprotein-cholesterol (LDL-C), tumor necrosis factor alpha (TNF-α,), interleukin 6 (IL6) and malondialdehyde (MDA) levels were significantly decreased in the TQ treated diabetic animals as compared to diabetic control group. On the other hand, TQ administered to diabetic rats led to significant increase in high density lipoprotein-cholesterol (HDL-C), total protein, glutathione reduced form (GSH), superoxide dismutase (SOD), catalase (CAT), adiponectin, hemoglobin (Hb), packed cell volume (PCV) as compared to diabetic control rats.

Conclusion: TQ significantly improved antioxidant status and reduced lipid peroxidation in blood, liver, pancreas and kidney tissues of diabetic treated rats through its anti-inflammatory, antioxidative and antiperoxidative properties.

Keywords: Diabetes, Thymoquinone, Oxidative stress, Inflammatory

Address for Correspondence

Department of Pharmacology, National Research Centre, Egypt.
E-mail: Bashandys @hotmail.com

markers, Liver and kidney functions, Hypolipidemic.

INTRODUCTION

Natural Products traditionally have played a drug discovery and they were the basis of most early medicines. They were used in folk medicine for the treatment of many diseases and illnesses[1]. Various plants have been found to possess significant antidiabetic property. Moreover, during the past few years many phytoconstituents responsible for antidiabetic effects have been isolated from plants[2]. Thymoquinone (TQ) (2-isopropyl-5-methyl-1, 4-benzo-quinone) is the bioactive phytochemical constituent of the volatile oil of *Nigella sativa* seeds[3]. TQ is a relatively safe compound, particularly when given orally to experimental animals[4]. TQ has been reported to exhibit many pharmacological effects, including immuno-modulatory[5], anticancer[6], antidiabetic[7], antioxidant, and anti-inflammatory activities[8]. TQ regulates the plasma concentrations of cholesterol, triglycerides, and glucose[9]. Previous study showed that TQ has a significant protective action toward an array of free radical creating compounds like doxorubicin induced cardiotoxicity[10], cisplatin-induced hepato-toxicity[11] and cadmium induced reprotoxicity[12]. Moreover, TQ has protective effect against both diabetic nephropathy[13] and membrane induced lipid peroxidation[14].

Diabetes mellitus is a serious metabolic disorder which is a major source of ill health all over the world and its incidence is expected to increase by 5.4% in 2025[15]. It has been shown that patients with diabetes mellitus have increased oxidative stress and impaired antioxidant defense systems, which appear to contribute to the initiation and progression of diabetes-associated complications [16]. It has been reported that streptozotocin (STZ) acts as a diabetogenic agent owing to its ability to destroy pancreatic β-cells, possibly by a free radical mechanism[17]. The level of lipid peroxidation in cell is controlled by various cellular defense mechanisms consisting of enzymatic and non-enzymatic scavenging systems which are altered in diabetes[18]. Moreover, disturbances of antioxidant defense systems in diabetes showed alteration in antioxidant enzyme levels, such as superoxide dismutase (SOD) and catalase (CAT), along with impaired glutathione (GSH) metabolism[19]. Antioxidants, a free radical scavengers may help in the regeneration of β-cell and protect pancreatic islets against cytotoxic effect of STZ[20]. Considering the various beneficial effects of TQ, this study was designed to evaluate alleviation of diabetes associated complications as hyperlipidemia, hepatitis and nephropathy through controlling oxidative stress and inflammatory markers by TQ.

MATERIALS AND METHODS

Chemicals

Thymoquinone, STZ and thio-barbituric acid were purchased from Sigma Chemical Co. (St. Louis Mo, USA). All other chemicals used were of analytical grade.

Animals

Male Wistar albino rats (*Rattus norvegicus*), weighing 180–200 g, were obtained from the animal house, National Research Centre, Egypt. The animals were housed throughout the experiment in polypropylene cages (each cage housing five animals) and allowed to acclimatize to laboratory environment for seven days before the beginning of the experiment.

Animals were maintained under controlled conditions of temperature (25 °c± 1 °c), humidity (50±15%) and normal photoperiod (12–12 h light-dark cycles). Rats had free access to standard rodent chow and water *ad libitum*. All animals received human care in compliance with guidelines of Ethical Committee of National Research Centre and followed the recommendations of The National Institute of Health Guide for care and use of Laboratory animals (Eighth edition).

Induction of diabetes

Experimental diabetes was induced by single intraperitoneal injection of streptozotocin (50 mg /kg) dissolved in 0.1 M of cold citrate buffer (pH 4.5). Because STZ is capable of inducing fatal hypoglycemia as a result of massive pancreatic insulin release, the rats were provided with 10% glucose solution after 6 h of STZ administration to prevent hypoglycemia. Neither death nor any other adverse effect was observed. After three days in time for the development of diabetes, rats with moderate diabetes (i.e. blood glucose concentration, >250 mg/ dl) were selected for the experiment (Zero time), while rats with blood glucose levels lower than the previous level were excluded from the study. All treatments were carried out three days after STZ had been injected. The weight of animals was recorded weekly for three weeks.

Experimental design

Rats were randomly divided into 3 equal groups (10 rats each) as follows:

Group I: Control group received vehicle only.

Group II: STZ group (Diabetic control group).

Group III: Diabetic group treated with TQ at dose level of 40mg/Kg bw which equal to 1/20 of LD_{50}.TQ was dissolved in corn oil and gavaged orally by stomach tube daily for three weeks. It was reported that LD_{50} of TQ was 794.3 mg/kg for oral gavage[21]. 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.

Samples collection

Blood samples from each group, were collected by puncture retro-orbital venous sinus, into heparinized tubes under light ether anesthesia weekly. The blood was centrifuged at 3500 rpm for 10 min to separate plasma which stored at -40°C.After removing the plasma, the packed RBCs were washed twice with cold isotonic physiological saline solution, then a known volume of RBCs was lysed in cold phosphate buffer (at pH=7.4). The haemolysate was separated by centrifuging at 3500 rpm for10 min, at 2˚C.Both plasma and haemolysate were used for biochemical analysis. Blood glucose and insulin levels were determined weekly, while other parameters were determined at the end of the experimentation period.

After blood samples collection, the animals from all groups were autopsied under light ether anesthesia. Liver, kidney and pancreas were removed from surrounding tissues and placed into tubes. The organs were dried between two sheets of filter paper, washed with cold normal saline and kept at -40°C. The tissues were homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4) and centrifuged at 3000 rpm for 10 min at 4 °C.

Blood cells count

Blood cells count and hemoglobin were determined by using hematology analyzer, Scil Vet ABC, operations manual, USA.

Biochemical variables assay

The resulting supernatant of both haemolysate and tissue homogenate was used for determination of reduced glutathione, catalase and superoxide dismutase levels using colorimetric assay kits according to the recommendations of the manufacturer (Biodiagnostic, Egypt). Moreover, Lipid peroxidation (LPO) was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a product formed due to the peroxidation of membrane lipids[22]. Blood hydroperoxide level was evaluated using an analytical system (Iram, Parma, Italy). The test is a colorimetric test that takes advantage of the ability of hydroperoxide to generate free radicals after reacting with transitional metals, when buffered chromogenic substance is added; a colored complex appears. This complex was measured spectrophotometrically. Also, the level of tumor necrosis factor-α, adiponectin (R&D Systems, USA) and interleukin 6 (IBL Germany) in plasma were determined by enzyme-linked immunosorbent assay (ELISA) using immunoassay kits according to the recommendations of the manufacturer. Plasma cholesterol, triglycerides, HDL, LDL, AST, ALT, urea, protein and creatinine were evaluated spectrophotometrically using diagnostic kits (Salucea, Netherland.)

Statistical analysis

All values were expressed as mean ± S.E. Statistical analysis of data was performed using two way Anova followed by least significant difference (LSD) for comparison of various treatments using the spss 13.0.

RESULTS

Effect of TQ on hyperglycemia

It is clear from table1 that the plasma glucose level of control diabetic rats increased significantly at the all time intervals of the experiment as compared with normal control. The plasma glucose levels of TQ-treated diabetic rats were significantly ($p \leq 0.01$) reduced by 27% and 45% at second and third weeks in comparison with the control diabetic rats. On the other hand, the insulin level of all treatment groups showed a significant decrease ($p \leq 0.01$)at all time intervals as compared to normal control, but the values of TQ-treated diabetic rats were significantly higher than those of control diabetic rats. The normal rats gained weight significantly during the experimental period, while in the control diabetic animals showed a significant decrease in body weight compared with the normal rats (Table 1). In contrast, TQ-treated diabetic rats alleviated the decrease in body weight.

Effect of TQ on blood cells

The results concerning blood cells count were represented by Fig. 1. RBC count of diabetic control rats (4.62±0.16 x 10^6/ml) showed a significant decrease ($p \leq 0.01$) as compared to normal control rats (6.95±0.25 x 10^6/ml). Diabetic rats treated with TQ showed a detectable amelioration of RBC count (5.87±0.11 x10^6/ml). The recorded values of diabetic rats showed a notable ($p \leq 0.01$) decrease in blood hemoglobin content (11.65±0.26g/dl) as compared with the normal control rats (16.21 ± 0.16 g/dl). Treatment with TQ elevated blood hemoglobin content of diabetic rats (14.22 ± 0.08) as compared with diabetic control rats. The significant decrease in the level of PCV (%) of the diabetic control animals (31.80±2.55) was observed as compared with normal control rats (48.36±2.05).TQ treatment led to improvement in the level of PCV (42.91±1.82). The present data showed a significant increase ($p \leq 0.01$) in WBCs count of diabetic group when compared with

control group, while TQ treatment inhibited this increase.

Effect of TQ on oxidative stress parameters

Figure 2 and table 2 showed the effects of TQ treatment on tissues (liver, kidney, pancreas) and blood oxidative stress in diabetic rats. Thiobarbituric acid reactive substances (TBARS) level of diabetic control rats either in tissues (liver, Kidney, Pancreas) or in RBCs elevated significantly as compared with normal control. Also, the blood hydroperoxide concentration increased significantly in diabetic rats. Administration of TQ to diabetic rats significantly decreased TBARS and hydroperoxide levels. TQ treatment significantly reduced the depletion in the activities of SOD, CAT and GSH content of tissues and RBCs of diabetic rats.

Effect of TQ on inflammatory markers

TQ administration minimized the significant ($P \leq 0.01$) increase in plasma TNF-α observed during diabetes (Fig. 3). The results of plasma interleukin 6 and adiponectin are showed in Figure 4. STZ treatment resulted in a significant increase in IL6 concentration and a significant decrease in adiponectin level. TQ treatment attenuated these effects.

Effect of TQ on hyperlipidemia

Table 3 shows the lipid profile and atherogenic index in normal and experimental animals in each group. The treatment of diabetic rats with TQ resulted in a significant decrease ($P \leq 0.01$) in the levels of plasma TC, TG, LDL-C and atherogenic index as compared with diabetic control rats. The significant decrease in HDL-C of diabetic rats was ameliorated by TQ.

Effect of TQ on liver and kidney function tests

Our results showed that STZ-induced diabetes caused a significant increase in plasma AST, ALT, urea and creatinine levels (Table3).On the other hand, plasma protein level of diabetic rats exhibited a significant decrease. TQ treatment attenuated these effects.

DISCUSSION

Traditional medicine or complementary and alternative medicines are effective sources of future drugs to counteract metabolic syndrome including diabetes. Natural products and their derivatives have historically been invaluable as a source of therapeutic agents[23]. Some natural compounds as TQ possessing antioxidant actions contribute to protection from oxidative stress-induced pathogenesis of diseases[24]. Accordingly, the present study was initiated to investigate antioxidative efficacies of TQ in STZ induced oxidative stress, inflammation, renal and hepatic dysfunction. In the present study, oral administration of TQ (40 mg/kg bw) to diabetic rats significantly decreased blood glucose and increased plasma insulin level. It was reported that TQ administration significantly improved glucose homeostasis through modification of activities of key enzymes of carbohydrate via enhanced insulin secretion in STZ-induced diabetic rats[25]. Antidiabetic action of thymoquinone is at least partially mediated through a decrease in hepatic gluconeogenesis[26] or via nitric oxide (NO) inhibitory pathway which involved in the destruction of β-cells during the development of diabetes mellitus[27]. Maintenance of normal cellular functions in the presence of free radicals largely depends on the efficiency of the defense mechanisms against reactive oxygen species (ROS)-mediated oxidative stress. Glutathione is considered to be the first line of cellular

defense against mediated oxidative damage. GSH functions by scavenging free radicals and consequently convert to its oxidized form, glutathione disulfide[28]. Superoxide dismutase is an important defense enzyme that catalyses the dismutation of superoxide radicals .Catalase is a hemoprotein that catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals[29]. TQ could act as a free radical and superoxide radical scavenger, as well as preserving the activity of various antioxidant enzymes such as catalase, glutathione peroxidase, and glutathione-S-transferase[30.] The findings of the present study indicated that TQ alleviated STZ-induced oxidative stress and β-cell damage in diabetic rats as manifested by a decrease in pancreatic MDA and increase of pancreatic SOD, Catalase and GSH levels. The present study indicated that TQ administration to diabetic rats lowered pancreatic oxidative stress which may lead to improvement of insulin level that result in hypoglycemic effect. Moreover, the present results demonstrate that TQ reduced the elevation of TNF-α in diabetic rats, a potential mediator of β-cell destruction in insulin--dependent diabetes mellitus[31]. Thymoquinone possess excellent antioxidant properties and could serve as a free radical scavenger, and this justifies its uses in alternative medicines[32]. Diabetic animals exhibit high oxidative stress due to persistent and chronic hyperglycemia, thereby depleting the activity of oxidative defense system, and thus promoting *de novo* free radical generation[33]. Oxidative stress has recently been shown to be responsible, at least in part, for pancreatic β-cell dysfunction caused by glucose toxicity. Under hyperglycemia, production of various reducing sugars, such as glucose-6-phosphate and fructose, increases through glycolysis and polyol pathways. During this process, reactive oxygen species (ROS) are produced and cause tissue damage[34-36]. Oxidative stress, implicated in the pathogenesis of a wide range of clinical disorders, refers to imbalance between the production of free radicals and the ability of the cells to defend against them. Oxidative stress can thus occur when the generation of free radicals increases, or the capacity to scavenge free radicals and repair of oxidatively modified macro-molecules decreases, or both[37].

Anemia in diabetes mellitus has been reported due to the increased non-enzymatic protein glycosylation of RBC membrane, which correlates with hyperglycemia[38]. Oxidation of this protein and hyperglycemia in Diabetes mellitus cause an increase in the production of lipid peroxides that lead to hemolysis of RBCs[39]. In this study, RBCs count, Hb concentration, PCV, and MCV of diabetic rats decreased significantly. Following TQ administration, RBC count and its related indices were appreciably improved that may be attributed to its ability to lower lipid peroxidation level that causes hemolysis of erythrocytes[40]. Moreover, TQ treatment to diabetic rats led to increase in blood GSH, SOD and catalase indicating antioxidant activity of TQ that alleviate damage in RBC caused by STZ.

Oxidative stress has been proposed to be involved in the pathophysiology of many chronic diseases, including atherosclerosis[41]. Our results revealed a significant increase in HDL with a concurrent significant reduction in TC, TG, LDL, and LDL/HDL ratio of diabetic rats administered TQ. The level of serum lipid profiles are usually raised in diabetic rats and such elevation represents a risk factor for coronary heart diseases[42]. Lowering the serum lipid level through dietary or drug therapy seems to be associated with a decrease in the risk of vascular disease. TQ has different mechanisms to exert its hypolipidemic effect. It was reported that

TQ ameliorated hyperlipidemia via reduction of hydroxyl-methyl-glutaryl COA reductase, controlling enzyme for Cholesterol biosynthesis[43]. The results of the present study indicated that administration of TQ to diabetic rats prevented the decrease in adiponectin level. Findings from various studies indicate a positive correlation between circulating adiponectin level and HDL cholesterol concentration[44,45]. HDL reverse cholesterol transport where by cholesterol synthesized is returned to the liver for reuse or re-excretion into the bile resulting in a decrease of cholesterol level[46]. An inverse relationship has been shown to exist between TNF-α and adiponectin. This effect is bi-directional, that is, primary changes in TNF-α can influence adiponectin concentrations and vice versa. TNF-α suppresses the expression and secretion of adiponectin from murine and human adipocytes in cell cultures[47].

MDA or release of cytokines such as TNF-α can promote leukocytes activation via induction of inflammatory pathways[48,49]. Our results revealed a significant increase in WBCs count, plasma TNF-α and IL6 levels of diabetic rats which ameliorated by TQ treatment. WBCs are potent producers of proinflammatory cytokines including IL-6[50]. It was reported that TQ has potential value in the treatment of inflammatory disease by reducing the levels of proinflammatory mediators[51]. Excessive oxidative stress has been implicated in the pathology and complications of diabetes mellitus[52]. TQ administration to present diabetic rats showed hepato-renal protective effects as evidenced by asignificant reduction in level of AST, ALT, urea and creatinine. Moreover, TQ treatment enhanced hepatic and renal antioxidant enzymes, SOD, CAT and GSH. Antioxidant enzymes form the first line of defense against ROS in the organism. The increased activities of antioxidant enzymes may act as an added compensation mechanism to maintain the cell integrity and protection against free radical damage induced by STZ.

The improvement of the heato-renal function associated with treating the diabetic rats with TQ could be attributed to its antidiabetic action resulting in alleviation of altered metabolic status in animals and to its potent antioxidant potential resulting in membrane stability. TQ is efficient cytoprotective agent against carbon tetrachloride or sodium fluoride induced hepatotoxicity through inhibition of the production of oxygen free radicals that cause lipid peroxidation[53,54] TQ also attenuates cypermethrin induced hepato-renal toxicity through oxidative stress [55].

From the above findings, we conclude TQ has the ability to ameliorate oxidative stress in plasma and tissues of STZ induced diabetic rats as evidenced by improved glycemic and antioxidant status of pancreas, liver, kidney and blood along with decreased lipid peroxidation and inflammatory markers. Thus, TQ should be considered as a treatment strategy for diabetic complications.

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Table 1. Effect of TQ on plasma glucose & insulin levels, and body weight of diabetic rats

Parameter	Treatment Time in week	Control	Diabetic	Diabetic + TQ
Glucose (mg/dl)	0	87.85±2.93	372.03±12.98**	411.21±14.12**
	1	92.67±1.87	384.23±8.71**	359.83±7.44**
	2	96.31±4.77	406.10±11.19**	294.54±13.21**@
	3	86.17±1.95	391.44±8.26**	214.60 ±8.77**@
Insulin (µU/ml)	0	17.43±0.23	6.17±0.24**	6.85±0.13**
	1	14.79±0.65	6.92±0.43**	7.54±0.21**
	2	15.34±0.76	5.83±0.22**	8.22±0.36**@
	3	16.46±0.50	5.63±0.31**	8.00±0.19**@
Body weight (g)	0	174.10± 8.70	183.40±9.40	189.8±6.40
	1	190.36±7.90	171.53±4.87	203.66±3.85
	2	244.11±10.76	200.32±6.22	240.75±8.24
	3	295.60±13.20	176.6±10.5**	254.6±11.5*@

All numbers are mean + standard error, n=10.
* Significantly different from control value,* $p<0.05$, **$p<0.01$.
@Significantly different from diabetic group value, @$p<0.01$.

Table 2. Oxidative stress parameters in tissues and blood of diabetic rats treated with TQ

Parameter \ Treatment	Control	STZ	STZ+TQ
Tissue MDA (nmol/g protein)			
Pancreas	0.86 ± 0.04	4.32 ± 0.12**	1.87 ± 0.03**@
Liver	3.59 ± 0.16	10.58 ± 0.49**	6.21 ± 0.32**@
Kidney	1.80 ± 0.03	6.11 ± 0.12**	2.16 ± 0.09**@
Plasma MDA (nmol/ml)	2.50 ± 0.16	4.62 ± 0.26**	3.05 ± 0.19*@
Tissue GSH (nmol/g tissue)			
Pancreas	12.93 ± 0.67	4.68 ± 0.36**	10.19 ± 0.42**@
Liver	19.44 ± 0.53	7.26 ± 0.45**	11.73 ± 0.81**@
Kidney	21.61 ± 1.12	5.39 ± 0.16**	15.38 ± 0.72**@
Blood GSH (mg/dl)	36.41 ± 1.15	21.64 ± 0.85**	30.52 ± 1.08**@
Tissue SOD (U/mg protein)			
Pancreas	11.39 ± 0.52	4.14±0.28**	9.01±0.30**@
Liver	16.76 ± 0.65	9.14±0.38**	13.56±0.67**@
Kidney	19.83 ± 0.54	7.25±0.32**	14.88±0.61**@
RBC SOD (U/g Hb)	120.54 ± 7.50	44.21±1.41**	100.78±5.64**@
Tissue catalase (nmol H2O2/min/mg protein)			
Pancreas	45.35±1.05	26.33±1.83**	39.80 ±2.11*@
Liver	98.34±3.68	57.49±2.67**	78.98±2.85**@
Kidney	68.13±4.35	32.64±0.69**	46.03±1.22**@
RBC CAT (U/mg Hb)	280.00±11.53	90.64±1.86**	250.54 ±7.21*@

All numbers are mean ± standard error, n=10.
* Significantly different from control value,* $p<0.05$, **$p<0.01$.
@ Significantly different from diabetic group value, @$p<0.01$.

Table 3. Effect of TQ on plasma lipid profile, kidney and liver functions of diabetic rats

Treatment Parameter	Control	STZ	STZ+TQ
Cholesterol (mg/dl)	78.16±2.27	128.58±2.37**	94.22±1.50**@
Triglycerides (mg/dl)	71.92±4.06	164.24±6.95**	107.28±2.68**@
HDL (mg/dl)	26.89±0.96	18.46±0.36**	22.20±0.55**@
LDL (mg/dl)	51.02±0.96	106.67±4.16**	73.17±3.68**@
LDL/HDL	1.93±0.09	5.82±0.25**	3.16±0.15**@
AST (u/L)	51.85±3.18	163.38±7.96**	107.44±2.46**@
ALT (u/L)	31.95±0.99	136.27±6.75**	79.58±2.84**@
Protein (g/dl)	7.30±0.28	5.84±0.12**	6.36±0.13**
Urea (mg/dl)	16.86±0.59	37.15±1.28**	26.13±1.14**@
Creatinine (mg/dl)	0.45±0.02	0.88±0.01**	0.59±0.02**@

All numbers are mean + standard error, n=10.
* Significantly different from control value, **p<0.01.
@ Significantly different from diabetic group value, @p<0.01.

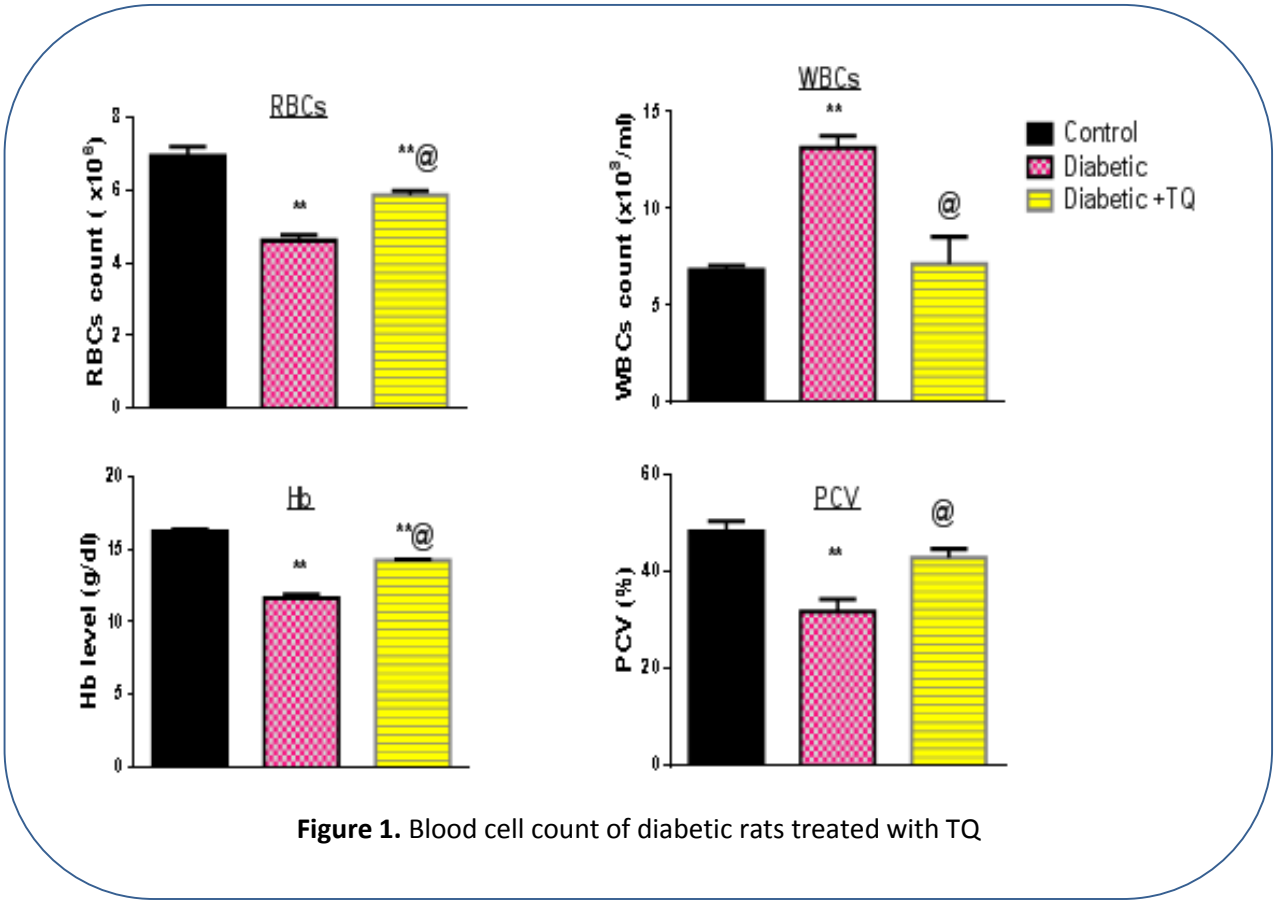

Figure 1. Blood cell count of diabetic rats treated with TQ

All numbers are mean + standard error, n=10.
* Significantly different from control value, **p<0.01.
@ Significantly different from diabetic group value, @p<0.01.

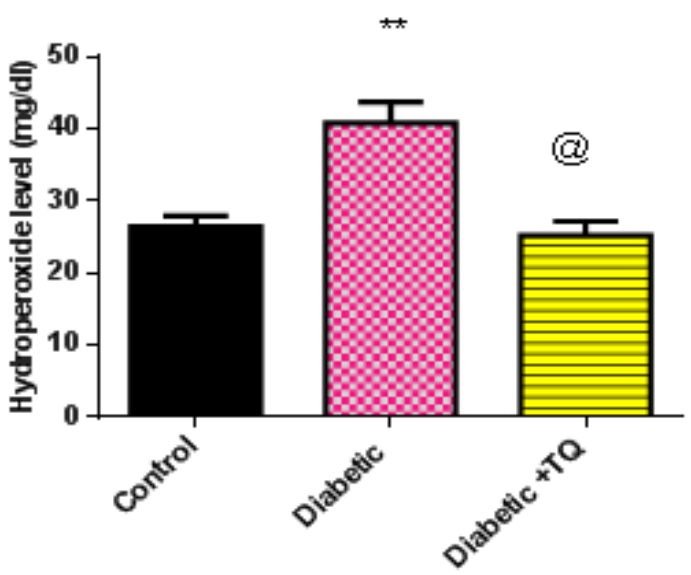

Figure 2. Blood hydroperoxide level of diabetic rat treated with TQ

Vertical bars represent mean + SEM from 10 replicate
(**p = 0.01 vs control; @p = 0.01 vs diabetic)

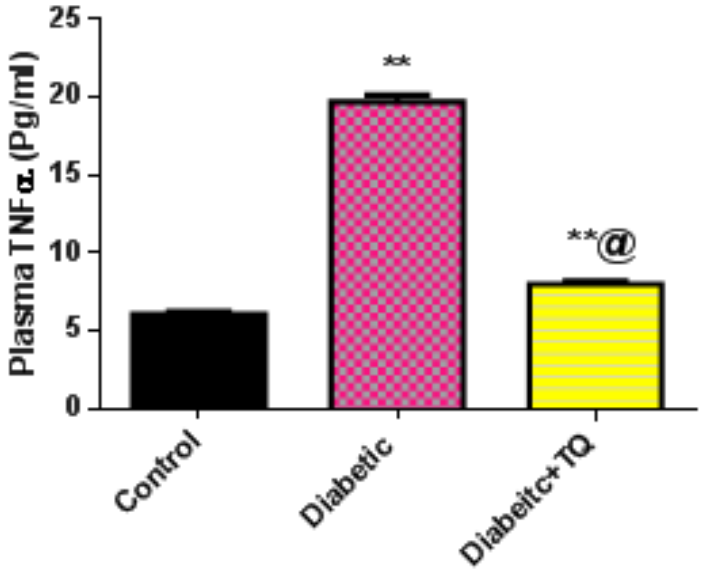

Figure 3. Plasma TNF-α of diabetic rats treated with TQ

Vertical bars represent mean + SEM from 10 replicate
(**p = 0.01 vs control; @p = 0.01 vs diabetic)

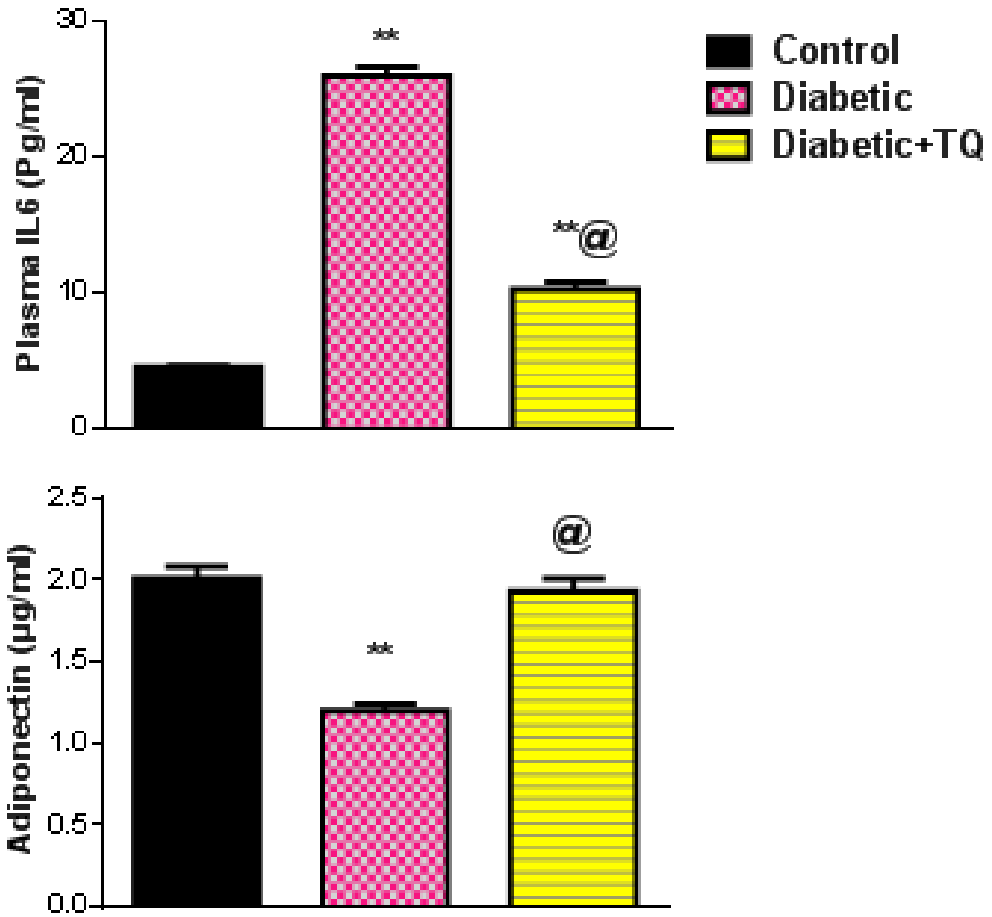

Figure 4. Plasma concentration of IL6 and adiponectin of diabetic rats treated with TQ

Vertical bars represent mean + SEM from 10 replicate
(**p = 0.01 vs control; @p = 0.01 vs diabetic)