Nitric oxide and endothelium-dependent effect of *Tribulus terrestris* feeding on aortic reactivity of streptozotocin-diabetic rats

Mehrdad Roghani[1*] **, Batool Rahmati**[1]**, Mohammad Reza Jalali-Nadoushan** [2] **, Mohammad-Reza Vaez Mahdavi** [3] **, Niusha Andalibi** [4]

1. Neurophysiology Research Center and Dept. Physiology, Shahed University, Tehran, Iran.
2. Department of Physiology, School of Medicine, Shahed University, Tehran, Iran.
3. Department of Pathology, School of Medicine, Shahed University, Tehran, Iran.
4. MD graduate from Shahed University, Tehran, Iran.

A B S T R A C T

Background and Objective: Cardiovascular disorders continue to constitute major causes of morbidity and mortality in diabetic patients. In this study, the effect of chronic administration of *Tribulus terrestris* (TT) feeding was studied on aortic reactivity of streptozotocin (STZ)-diabetic rats and some underlying mechanisms were investigated.

Materials and Methods: Male diabetic rats received TT-mixed food at a weight ratio of 6% for 7 weeks 1 week after diabetes induction. Contractile responses to KCl and phenylephrine (PE) and relaxation response to acetylcholine (ACh) were obtained from aortic rings in the presence and absence of endothelium. In addition, nitric oxide synthase inhibitor N(G)-nitro-l-arginine methyl ester (L-NAME) was used to determine the role of NO.

Results: Maximum contractile response of endothelium-intact rings to PE was significantly lower in TT-treated diabetic rats relative to untreated diabetics and endothelium removal abolished this difference. Endothelium-dependent relaxation to ACh was also significantly higher in TT-treated diabetic rats as compared to diabetic ones and pretreatment of rings with L-NAME significantly attenuated the observed response.

Conclusion: Chronic treatment of diabetic rats with TT could prevent some abnormal changes in vascular reactivity in diabetic rats through nitric oxide in aortic tissue and endothelium integrity is necessary for this beneficial effect.

1. Introduction

Cardiovascular disorders continue to constitute major causes of morbidity and mortality in diabetic patients in spite of significant achievements in their diagnosis and treatment(1). Changes in vascular responsiveness to vasoconstrictors and vasodilators are mainly responsible for development of some vascular complications of diabetics (2). Most of these complications are due to increased serum glucose and augmented generation of reactive oxygen species (ROS) which finally lead to endothelium dysfunction (3).

Tribulus terrestris L. is a member of the Zygophyllaceae family. It is an annual herb about 30–70 cm high and has pinnate leaves (of unequal length), yellow flowers and characteristic stellate shaped carpel fruits. It is widely distributed in

Corresponding Author:
Mehrdad Roghani
Department of Physiology, School of Medicine, Shahed University, Tehran, Iran
mehjour@yahoo.com

Africa, western Asia, China, Japan, Korea and Europe (4). Extracts from this plant have been used traditionally in treating a variety of diseases including hypertension and coronary heart disease, ocular inflammation and infertility in both sexes. The extracts have also been used as diuretics (4). Recent pharmacological studies tend to support these uses. For example, Al-Ali et al. (2003) have demonstrated diuretic activity in rats (5), while Adaikan et al. (2000) have shown that crude extract of *Tribulus terrestris* enhanced electrically- and nitroglycerine- induced relaxation of the rabbit corpus cavernosum consistent with a pro-erectile function (6). The mechanism responsible for the antihypertensive activity has not fully been understood (4). In a recent study, Sharifi et al. reported a significant antihypertensive effect of an aqueous extract of *Tribulus terrestris* in renin-dependent 2-kidney 1-clip (2K-1C) model of hypertension and suggested that this might be related to its inhibitory effect on angiotensin converting enzyme (ACE) activity (7). This was based on the observation that treatment with the aqueous extract *Tribulus terrestris* significantly reduced ACE activity in all tissues of the rat (7). This study was designed to assess for the first time the beneficial effect of chronic *Tribulus terrestris* feeding on improvement of aortic reactivity dysfunction of STZ-diabetic rats and to investigate some underlying mechanisms.

2. Materials and Methods

2.1. Animals

Male albino Wistar rats (n=28) (Pasteur's institute, Tehran, Iran) weighing 225-310g were housed in an air-conditioned colony room at 21 2C and supplied with standard pellet diet and tap water ad libitum. Procedures involving animals and their care were conducted in conformity with NIH guidelines for the care and use of laboratory animals.

2.2. Experimental protocol

The rats were rendered diabetic by a single intraperitoneal dose of 60 mg kg-1 STZ freshly dissolved in ice-cold 0.1 M citrate buffer (pH 4.5). Age-matched normal animals that received an injection of an equivalent volume of buffer comprised a non-diabetic control group. One week after STZ injection, overnight fasting blood samples were collected and serum glucose concentrations were measured using glucose oxidation method (Zistchimie, Tehran). Only those animals with a serum glucose level higher than 250 mg/dl were selected as diabetic. During the next weeks, diabetes was reconfirmed by the presence of polyphagia, polydipsia, polyuria, and weight loss. Normal and hyperglycemic rats (a total of 48) were randomly allocated and similarly grouped into 4 groups (seven in each): normal vehicle-treated control, *Tribulus terrestris* (TT)-treated control, diabetic, and TT-treated diabetic. TT powder was mixed with standard food at a ratio of 6% and was freely available to rats throughout the experimental period for 7 weeks. Changes in body weight were regularly recorded during the study. The rats were finally anesthetized with diethyl ether, decapitated, and through opening the abdomen, descending thoracic aorta was carefully excised and placed in a petri dish filled with cold Krebs solution containing (in mM): NaCl 118.5, KCl 4.7, CaCl2 1.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, and glucose 11. The aorta was cleaned of excess connective tissue and fat and cut into rings of approximately 4 mm in length. Aortic rings were suspended between the bases of two triangular-shaped wires. One wire was attached to a fixed tissue support in a 50 ml isolated tissue bath containing Krebs solution (pH 7.4) maintained at 37 C and continuously aerated with a mixture of 5% CO_2 and 95% O_2. The other end of each wire attached by a cotton thread to a F60 isometric force transducer (Narco Biosystems, USA) connected to a computer. In all experiments, special care was taken to avoid damaging the luminal surface of endothelium. Aortic rings were equilibrated at a resting tension of 1.5 g for at least 45 min. In some experiments, the endothelium was mechanically removed by gently rubbing the internal surface with a filter paper. Isometric contractions were induced by the addition of phenylephrine (PE,$1\,\mu m$) and once the contraction stabilized, a single concentration of acetylcholine ($1\,\mu m$) was added to the bath in order to assess the endothelial integrity of the preparations. Endothelium was considered to be intact when this drug elicited a vasorelaxation $\geq 50\%$ of the maximal contraction obtained in vascular rings precontracted with PE. The absence of acetylcholine relaxant action in the vessels indicated the total removal of endothelial cells. After assessing the integrity of the endothelium, vascular tissues were allowed to recuperate for at least 30 min.

At the end of the equilibration period, dose–response curves with KCl (10-50 mM) and PE (10-10-10-5 M) in the presence and absence of endothelium were obtained in aortic rings in a cumulative manner. To evaluate Ach-(10-9-10-4 M) and SNP-(10-9-10-4) induced vasodilatation in rings with and without endothelium, they were preconstricted with a submaximal concentration of PE (10-6 M) which produced 70-80% of maximal response. To determine the participation of NO, rings were incubated 30 min before the experiment with L-NAME (100μM, a non-selective NOS inhibitor).

After each vasoreactivity experiment, aortic rings were blotted, weighed, and the cross-sectional area (csa) was calculated using the following formula: Cross-sectional area (mm2) = weight (mg) [length (mm) density (mg mm3-1)]-1. The density of the preparations was assumed to be 1.05 mg/mm2.

2.3. Drugs

Phenylephrine, streptozotocin, ACh and L-NAME were purchased from Sigma Chemical (St. Louis, Mo., USA). All other chemicals were purchased from Merck (Germany) and Darupakhsh Co. (Iran).

2.4. Data and statistical analysis

All values were given as means SEM. Contractile response to PE was expressed as grams of tension per cross-sectional area of tissue. Relaxation response for ACh was expressed as a percentage decrease of the maximum contractile response induced by PE. Statistical analysis was carried out using repeated measure ANOVA and one-way ANOVA followed by Tukey post-hoc test. A statistical p value less than 0.05 considered significant.

3. Results

After 8 weeks, the weight of the vehicle-treated diabetic rats was found to be significantly decreased as compared to controls ($p < 0.005$) and TT treatment at a ratio of 6% caused a significant lower reduction in diabetic rats as compared to vehicle-treated diabetics ($p < 0.05$). Untreated diabetic rats had also an elevated serum glucose level over those of control rats ($p < 0.0005$) and treatment of diabetic rats with TT at a ratio of 6% caused a significant decrease in the serum glucose relative to diabetics at week 8 ($p < 0.05$). In addition, TT treatment of control rats did not produce any significant change regarding serum glucose level (Fig.1).

Cumulative addition of KCl (10-50 mM) and PE (10-10-10-5 M) resulted in concentration dependent contractions in aortas of all groups (Figures 2-3). The maximum contractile responses to KCl and PE in the aortas from vehicle-treated diabetic rats in the presence of endothelium were found to be significantly ($p < 0.01$-0.005) greater than vehicle-treated control rats and concentration-response curve of endothelium-intact aortas from TT-treated diabetic rats at a ratio of 6% to PE (and not to KCl) was significantly attenuated compared to vehicle-treated diabetics ($p < 0.05$). Although endothelium-denuded aortic rings in all groups showed a higher contractile response to KCl and PE, but the observed changes between treated and untreated diabetics were attenuated after endothelium removal. This clearly indicates the necessity of endothelium presence for beneficial vascular effect of TT. In addition, aortic rings with endothelium from TT-treated control group showed a non-significant reduction in contractile response to KCl and PE as compared to vehicle-treated controls.

Addition of ACh resulted in concentration-dependent relaxations in all aortic rings precontracted with PE (Fig. 4). As was expected, endothelium-dependent relaxation responses induced by ACh was significantly lower in vehicle-treated diabetic rats in relation to vehicle-treated controls ($p < 0.05$-0.005). Meanwhile, the existing difference between TT-treated at a ratio of 6% and vehicle-treated diabetic rats was only significant ($p < 0.05$) at concentrations higher than 10-4 M. Meanwhile, relaxation response of TT-treated control rats was non-significantly greater than control group.

Pre-incubation of aortic rings with L-NAME non-significantly increased contractile response of aortic rings from all groups to PE. However, this increase was non-significantly lower in TT-treated diabetic group as compared to vehicle-treated diabetics. Regarding relaxation response to ACh, pre-incubation of aortic rings with L-NAME almost completely abolished the vasodilator response to ACh in segments from TT-treated diabetic rats, indicating the important role of endothelium-derived NO in the vascular effect of TT (Fig. 5).
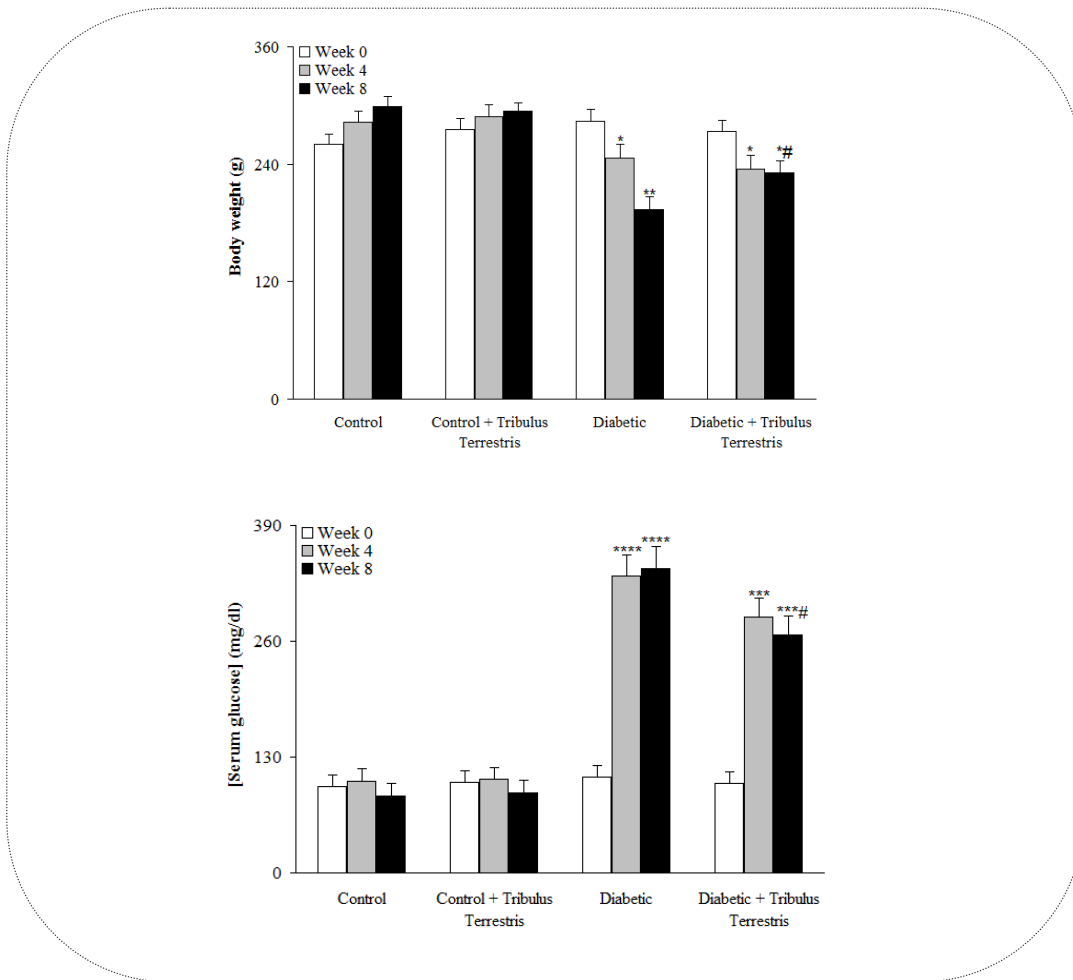
Fig. 1: Body weight and serum glucose concentration in different weeks (means ± S.E.M). * p<0.05, ** p<0.005, *** p<0.001, **** p<0.0005 (as compared to week 0 in the same group) # p<0.05 (Versus diabetic in the same week).
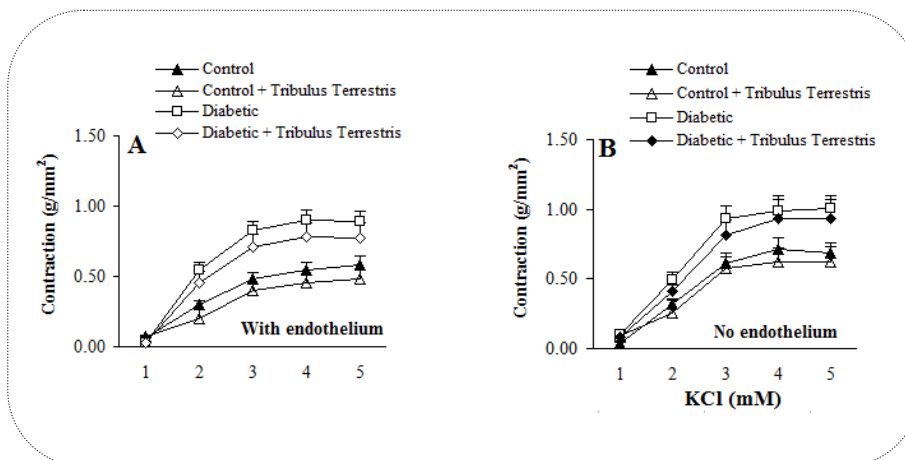
Fig. 2: Cumulative concentration-response curves for KCl in aortic preparations 8 weeks after experiment in the presence (A) and absence (B) of endothelium (means ± S.E.M).
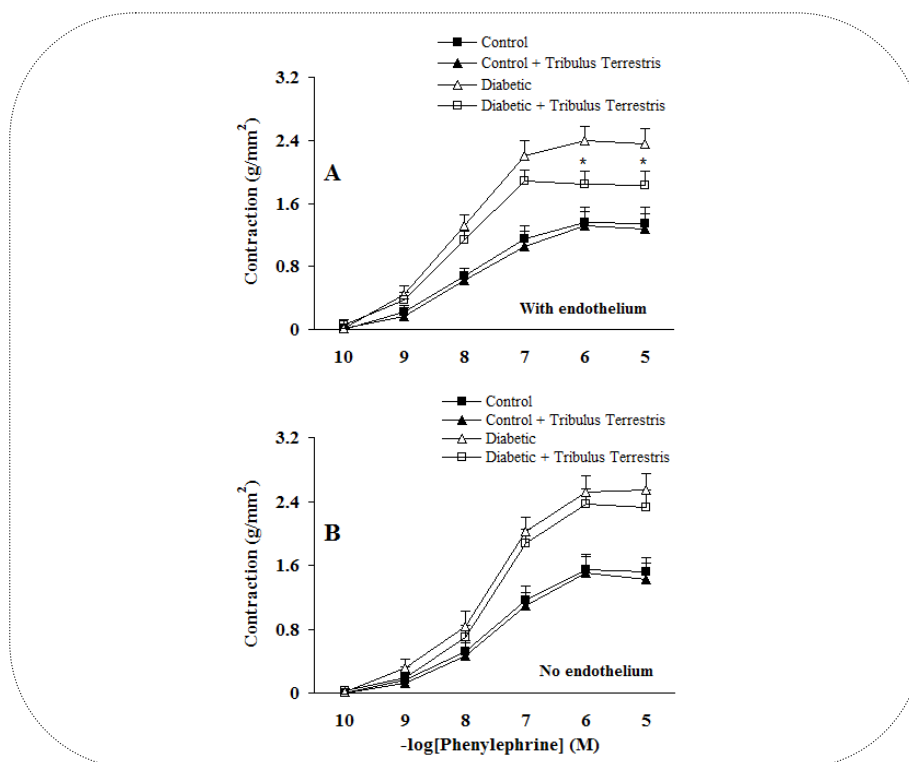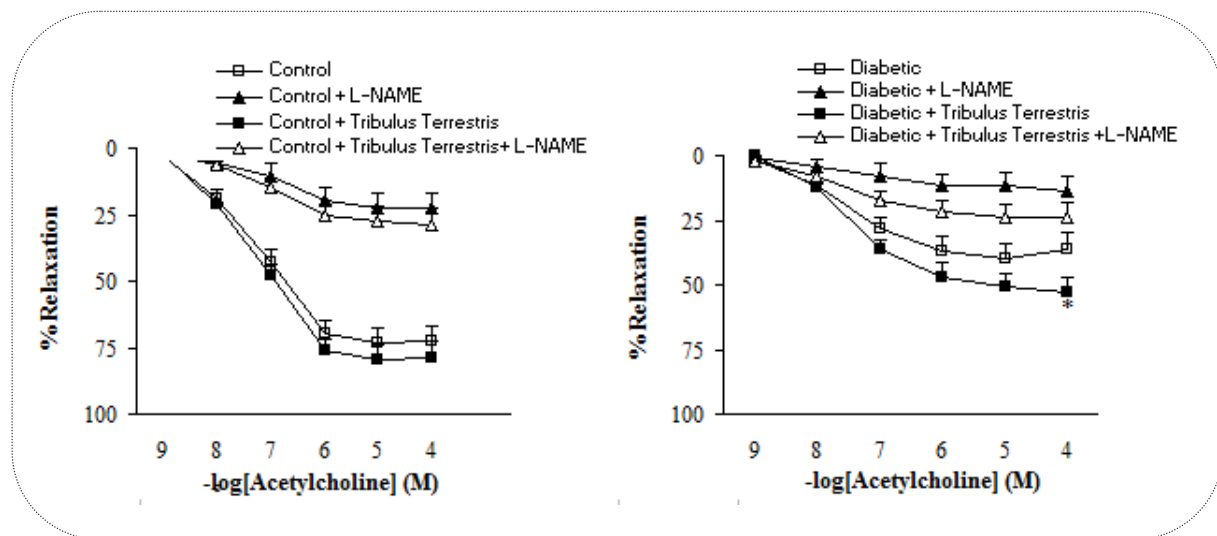
Fig. 3: Cumulative concentration-response curves for PE in aortic preparations 8 weeks after experiment in the presence (A) and absence (B) of endothelium (means ± S.E.M). * p<0.05 (as compared to diabetic)
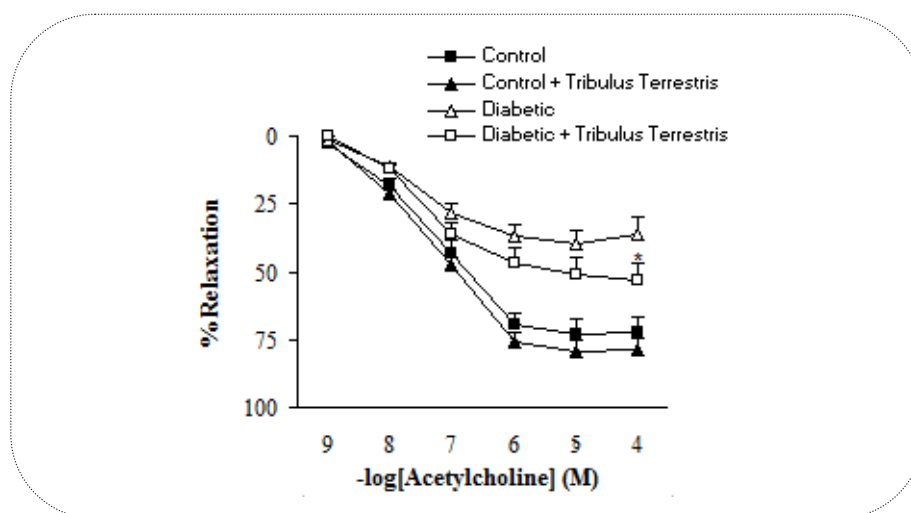
Fig. 4: Cumulative concentration-response curves for ACh in endothelium-intact aortic rings precontracted with PE 8 weeks after experiment. Relaxation responses are expressed as a percentage of the submaximal contraction induced by phenylephrine which produced 70-80% of maximal response (means ± SEM). * p<0.05 (as compared to diabetic)

Fig. 5: Cumulative concentration-response curves for ACh in endothelium-intact aortic rings precontracted with phenylephrine in the presence and absence of L-NAME 8 weeks after the experiment in control and diabetic rats. Relaxation responses are expressed as a percentage of the submaximal contraction induced by phenylephrine which produced 70-80% of maximal response (means ± SEM). * $p<0.05$ (as compared to diabetic)

4. Discussion

In this study, administration of *Tribulus terrestris* for 7 weeks did have a moderate hypoglycemic effect, it reduced the enhanced contractility of aortic rings to PE and increased ACh-induced relaxation which was partly due to involvement of NO pathway since the relaxation was blocked in the presence of L-NAME. In addition, endothelium removal clearly affected KCl- and PE-induced contractions in TT-treated diabetic rats.

Vascular dysfunction is one of the complicating features of diabetes in humans and its experimental model and hyperglycemia is the primary cause of micro and macrovascular complications in diabetic condition (8). Compared to the aortic rings from control animals, contraction of aortas to KCl and PE from diabetic rats significantly increased that was consistent with previous studies (9) and chronic TT was capable to attenuate this change only for PE-induced contractions. Impaired endothelial function (10), enhanced sensitivity of calcium channels (11), an increase in vasoconstrictor prostanoids due to increased superoxide anions and increased sensitivity to adrenergic agonists (12) might all be responsible for increased contractile responses in diabetic

rats, which could have been improved following TT treatment.

In endothelial cells of most vascular beds, ACh could stimulate production and release of endothelial-derived relaxing factors including nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor and in this way leads to relaxation of vascular smooth muscle in an endothelium-dependent manner (13-15). The ACh-induced relaxation response is endothelium-dependent and NO-mediated (9). The results of this work revealed that the endothelium-dependent relaxant response was reduced in aortas from STZ-induced diabetic rats and this reduced relaxation was partially recovered by TT treatment. Although some researchers asserted that the sensitivity to acetylcholine decreases in diabetes (12), the results of this research, in accordance with those of many previous ones (16) reveals that diabetes condition in long-term only decrease the maximum responses to ACh but not the sensitivity (pD2).

Impaired endothelium-dependent relaxation in STZ-induced diabetic rat might be due to increased blood glucose level and decreased blood insulin level. It has been shown that hyperglycaemia causes tissue damage with several mech-

anisms, including advanced glycation end product (AGE) formation, increased polyol pathway flux, apoptosis and reactive oxygen species (ROS) formation (17). Our results showed that TT treatment could exert a significantly weak hypoglycemic effect in STZ-induced diabetic rats, therefore, its beneficial effect on aortic tissue of diabetic rats should be in part due to its hypoglycemic effect. Some damaging effect of diabetes on vascular tissue of diabetic animals is also believed to be due to enhanced oxidative stress, as shown by enhanced MDA and decreased activity of defensive enzymes like SOD (18). This could also lead to diabetes-induced functional changes in vascular endothelial cells and the development of altered endothelium-dependent vasoreactivity.

In conclusion, to the best of our knowledge, this is the first study to report that in vivo chronic treatment of diabetic rats with *Tribulus terrestris* endothelium-dependently could prevent the functional changes in vascular reactivity observed in diabetic rats through nitric oxide. Our data may be helpful in the development of new natural drugs to improve endothelial function and to prevent cardiovascular diseases.

4.1. Acknowledgment

References

1. Coccheri S. Approaches to prevention of cardiovascular complications and events in diabetes mellitus. Drugs. 2007;67(7):997-1026.

2. Nasri S, Roghani M, Baluchnejadmojarad T, Rabani T, Balvardi M. Vascular mechanisms of cyanidin-3-glucoside response in streptozotocin-diabetic rats. Pathophysiology. 2011; 18(4):273-8.

3. Naito M, Fujikura J, Ebihara K, Miyanaga F, Yokoi H, Kusakabe T, et al. Therapeutic Impact of Leptin on Diabetes, Diabetic Complications, and Longevity in Insulin-Deficient Diabetic Mice. Diabetes. 2011; 60(9):2265-73.

4. Phillips OA, Mathew KT, Oriowo MA. Antihypertensive and vasodilator effects of methanolic and aqueous extracts of Tribulus terrestris in rats. J Ethnopharmacol. 2006;104(3):351-5.

5. Al-Ali M, Wahbi S, Twaij H, Al-Badr A. Tribulus terrestris: preliminary study of its diuretic and contractile effects and comparison with Zea mays. J Ethnopharmacol. 2003; 85(2-3):257-60.

6. Adaikan PG, Gauthaman K, Prasad RN, Ng SC. Proerectile pharmacological effects of Tribulus terrestris extract on the rabbit corpus cavernosum. Ann Acad Med Singapore. 2000;29(1):22-6.

7. Sharifi AM, Darabi R, Akbarloo N. Study of antihypertensive mechanism of Tribulus terrestris in 2K1C hypertensive rats: role of tissue ACE activity. Life Sci. 2003; 73(23):2963-71.

8. Madonna R, De Caterina R. Cellular and molecular mechanisms of vascular injury in diabetes--part I: pathways of vascular disease in diabetes. Vascul Pharmacol. 2011;54(3-6):68-74.

9. Roghani M, Baluchnejadmojarad T. Chronic epigallocatechin-gallate improves aortic reactivity of diabetic rats: underlying mechanisms. Vascul Pharmacol. 2009; 51(2-3):84-9.

10. Olukman M, Sezer ED, Ulker S, Sozmen EY, Cinar GM. Fenofibrate treatment enhances antioxidant status and attenuates endothelial dysfunction in streptozotocin-induced diabetic rats. Exp Diabetes Res. 2010;2010:828531.

11. Chang KC, Chung SY, Chong WS, Suh JS, Kim SH, Noh HK, et al. Possible superoxide radical-induced alteration of vascular reactivity in aortas from streptozotocin-treated rats. J Pharmacol Exp Ther. 1993;266(2):992-1000.

12. Abebe W. Effects of taurine on the reactivity of aortas from diabetic rats. Life Sci. 2008;82(5-6):279-89.

13. Zhang LN, Vincelette J, Chen D, Gless RD, Anandan SK, Rubanyi GM, et al. Inhibition of soluble epoxide hydrolase attenuates endothelial dysfunction in animal models of diabetes, obesity and hypertension. Eur J Pharmacol. 2011;654(1):68-74.

14. Flammer AJ, Luscher TF. Human endothelial dysfunction: EDRFs. Pflugers Arch. 2010;459(6):1005-13.

15. Takaki A, Morikawa K, Murayama Y, Yamagishi H,

Hosoya M, Ohashi J, et al. Roles of endothelial oxidases in endothelium-derived hyperpolarizing factor responses in mice. J Cardiovasc Pharmacol. 2008; 52(6):510-7.

16. Silan C. The effects of chronic resveratrol treatment on vascular responsiveness of streptozotocin-induced diabetic rats. Biol Pharm Bull. 2008; 31(5):897-902.

17. Hartge MM, Unger T, Kintscher U. The endothelium and vascular inflammation in diabetes. Diab Vasc Dis Res. 2007; 4(2):84-8.

18. Baluchnejadmojarad T, Roghani M. Chronic administration of genistein improves aortic reactivity of streptozotocin-diabetic rats: mode of action. Vascul Pharmacol. 2008; 49(1):1-5.