The role of nitric oxide and prostaglandins in the effects of alcoholic *Trigonella foenum-graecum* seed extract on aortic reactivity in streptozotocin-diabetic rats

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ABSTRACT

Background and the purpose of the study: Trigonella foenum-graecum (TFG) has demonstrated beneficial effects in both Insulin-dependen and non- Insulin-dependen diabetic animals. This study was conducted to evaluate the effects of the alcoholic seed extract of this plant on aortic reactivity and underlying mechanisms in streptozotocin-diabetic rats.

Methods: Male Wistar rats were divided into control, extract-treated control, diabetic, and extract-treated diabetic groups. Diabetes was induced by a single i.p. injection of streptozotocin (STZ; 60 mg/kg). Treatment groups received TFG extract (200 mg/kg; i.p.) every other day for 1 month. Then, contractile responsiveness of thoracic aorta to KCl and noradrenaline (NA) and relaxation to acetylcholine (ACh) and sodium nitroprusside (SNP) was determined. For determination of the involvement of NO and prostaglandins in relaxation response to ACh, rings were incubated 30 min before the experiment with N(ω)-nitro-L-arginine methyl ester (L-NAME) and/or indomethacin (INDO).

Results: Diabetic state significantly increased maximum contractile responses to KCl and NA ($p<0.01$-0.005) and reduced maximum relaxation due to ACh ($p<0.01$) as compared to controls and treatment with TFG extract in diabetic group significantly improved these changes relative to untreated diabetic group ($p<0.05$). Meanwhile, pretreatment with L-NAME did not produce any significant change between diabetic and extract-treated diabetic groups. On the other hand, there was a significant difference in both of these two groups following pretreatment with INDO ($p<0.01$).

Major conclusion: Intraperitoneal administration of alcoholic seed extract of TFG for one month could improve some functional indices of the vascular system in diabetic state and endothelium-derived prostaglandins are involved in this response.

Keywords: *Trigonella foenum-graecum*, Diabetes mellitus, Streptozotocin, Aorta

INTRODUCTION

Mortality from cardiovascular abnormalities including hypertension, atherosclerosis, microangiopathy, and congestive heart failure is about three times more prevalent in the diabetic population than in the general population (1-2). Therefore, finding new treatment strategies for attenuation of diabetic vascular complications has always been a main strategy in medicine. In this regard, *Trigonella foenum-graecum* (TFG; fenugreek) has been considered as an appropriate candidate. *TFG* is a plant with traditional medicinal use in diabetes. Beneficial effects have been demonstrated in diabetic animals and in both insulin-dependent and non-insulin-dependent diabetic subjects (3). Hypoglycemic and anti-hyperglycemic effects of fenugreek seed (4) and aqueous leaf extracts (5) have previously been reported in experimentally induced diabetic rats. In addition, endothelium-dependent attenuating effects of this plant in aortic rings from diabetic rats have previously been reported (6). The present study was carried out to evaluate the mechanisms responsible for beneficial effects of the extract of the seed of this plant on vascular reactivity of thoracic aorta from STZ-diabetic rats.

MATERIALS AND METHODS

Preparation of TFG extract

Fenugreek seed was obtained from local grocery (Tehran) in June and was systemically identified.

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Then, seeds were washed, dried under shade at room temperature, and finely powdered with a grinder. Thereafter, 100 g of powder was suspended in 1 L of methanol for 48 h in dark (maceration method). The extract was filtered and concentrated to obtain the solid residue and after determination of final weight, it was refrigerated until further use. The yield of this process was 19.5% (w/w). Fenugreek extract of lower concentrations was prepared by dilution of the stock with cold and sterile 0.9% saline solution.

Animal experiments

Male albino Wistar rats (Pasteur's institute, Tehran, Iran) weighing 245-285 g (10-12 weeks old) were housed in an air-conditioned colony room at 23 ± 1 °C 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 (7).

The animals (n = 30) were randomly divided into four experimental groups: vehicle-treated control (VC, n = 7), extract-treated control (EC, n = 7), vehicle-treated diabetic (VD, n = 7), and extract-treated diabetic (ED, n = 9). Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 60 mg/Kg) dissolved in cold 0.9% saline immediately before use. Control and extract-treated control animals received normal saline solution and alcoholic extract of fenugreek extract (200 mg/Kg, i.p.) respectively. This dose was chosen on the basis of our previous studies and other reports (6). The latter was administered every other day to extract-treated diabetic animals from the day of 3 after induction of hyperglycemia by STZ. Serum glucose level and body weight were measured one week before and four weeks after the experiment. Diabetes was verified by a non-fasting serum glucose level higher than 250 mg/dl using glucose oxidation method (glucose oxidase kit, Zistchimie, Tehran, Iran). All treatments continued for one month.

Experimental procedure

The routine protocol was applied as previously described (8-9). Briefly, after being anesthetized, descending thoracic aorta of the animal was carefully excised and placed in a petri dish filled with cold Krebs solution containing (mM): NaCl 118.5, KCl 4.74, $CaCl_2$ 2.5, $MgSO_4$ 1.18, KH_2PO_4 1.18, $NaHCO_3$ 24.9, and glucose 10.0 (8). The aorta was cleaned from the excess connective tissues and fat and cut into rings of approximately 4 mm in length. One ring of each pair was left intact, and for the other ring, endothelium was mechanically removed by gentle rotating on a glass micropipette. 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 5% O_2. The other end of each wire was attached by a cotton thread to a F60 isometric force transducer, which was connected to A/D board of the IBM-compatible computer. Recording and analysis of data was performed using the software Physiograph I (Behineh Arman Co., Tehran, Iran). The rings were allowed to equilibrate for 60 min under a resting tension of 2 g before beginning of experiments. During equilibration period, the rings were washed every 30 min. Successful removal of the endothelium was confirmed by loss of acetylcholine (10^{-5} M)-induced relaxation in preconstricted rings by NA (10^{-6} M). Concentration-response curves were obtained with KCl and thereafter with NA in aortic rings with or without endothelium. In this respect, KCl (10-50 mM) and NA (10^{-9}-10^{-4} M) were added in a cumulative manner until a maximum response was achieved. For endothelium-intact and endothelium-denuded segments, concentration-dependent relaxation response to ACh (10^{-9}-10^{-4} M) and sodium nitroprusside (SNP) (10^{-9}-10^{-4} M) were obtained respectively

To determine the involvement of NO in relaxation response, rings were incubated with L-NAME (100 μM, as a NOS inhibitor) 30 min before the experiment. To determine the participation of endothelial prostanoids in relaxation response, segments were incubated with 10 μM INDO, an inhibitor of COX-derived prostanoid synthesis, 30 min before the study.

After each experiment, aortic rings were dried at 45°C for 5 min, weighed, and cross-sectional areas (CSA) were calculated using the formula: Cross-sectional area (mm^2) = weight (mg) × [length (mm) × density (mg/mm^3)]$^{-1}$. The density of the preparations was assumed to be 1.05 mg/mm^3 (10).

Drugs and chemicals

Noradrenaline, acetylcholine-HCl, and SNP were purchased from Sigma Chemical (St. Louis, Mo., USA). Streptozotocin was obtained from Pharmacia and Upjohn (USA). All other chemicals were purchased from Merck (Germany) and Temad (Tehran, Iran). STZ was freshly dissolved in 0.9% saline solution. Indomethacin solution was prepared in ethanol in such a way that the maximal ethanol concentration of the medium was less than 0.001% (v/v).

Data and statistical analysis

All values were given as means ± SEM. Contractile responses to NA and KCl were expressed as grams of tension per cross-sectional area of tissue. Relaxation response is shown as percentage. Statistical analysis was carried out using student's paired t-test and one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. A statistical p value less than 0.05 considered significant.

RESULTS

Body weight, Serum glucose, and cross-sectional area

Body weight and serum glucose levels were measured before and 4 weeks after the experiment (Table 1). After one month, the body weight of the diabetic rats compared to body weigh of one week before the experiment ($p<0.05$) decreased significantly. In addition, a non-significant higher body weight was observed in extract-treated diabetic rats in comparison with diabetic group.

Diabetic rats had also elevated serum glucose levels compared with those of control rats ($p<0.001$). Treatment of diabetic rats with alcoholic extract of fenugreek (200 mg/kg) lowered serum glucose levels significantly ($p<0.01$). While, there was a significant reduction in cross-sectional area of aortic rings in diabetic group ($p<0.05$), extract-treated diabetic group did not show any significant improvement (data not shown).

Contractile responses to KCl and NA

Cumulative addition of KCl (10-50 mM) and NA (10^{-9}-10^{-4} M) to the organ bath resulted in concentration-dependent contractions in aortas of all groups (Figures 1 and 2). The contractile responses to KCl at concentrations higher than 20 mM in diabetic rats were found to be significantly higher than control rats both in the presence (Fig. 1A) or absence of endothelium (Fig. 1B) and treatment of diabetic rats with TFG caused a significant reduction in contractile response to KCl at a concentrations of 50 mM and only for endothelium-intact rings.

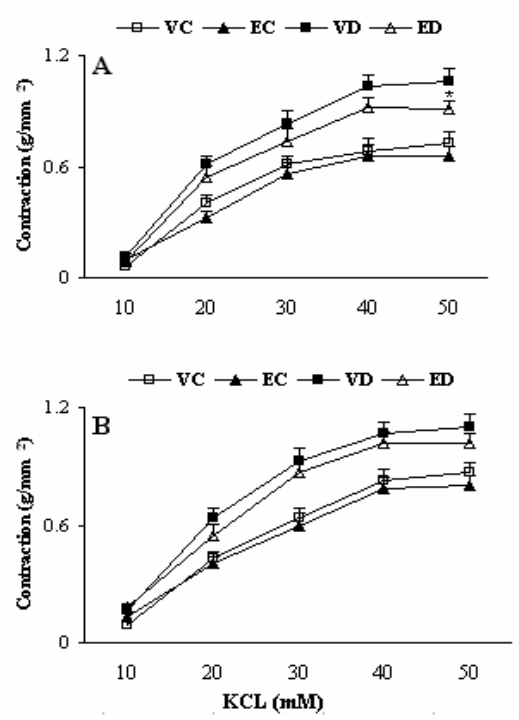


Figure 1. Cumulative concentration-response curves for KCl in aortic preparations one month after the experiment in the presence (A) or absence (B) of endothelium. Contractile responses are expressed as grams of tension per cross sectional area (mm^2). Data are shown as means ± SEM.

* P<0.05 (Compared to VD)(VC, EC, VD, and ED represent vehicle-treated control, extract-treated control, vehicle-treated diabetic, and extract-treated diabetic rats at a dose of 200 mg/kg of the extract).

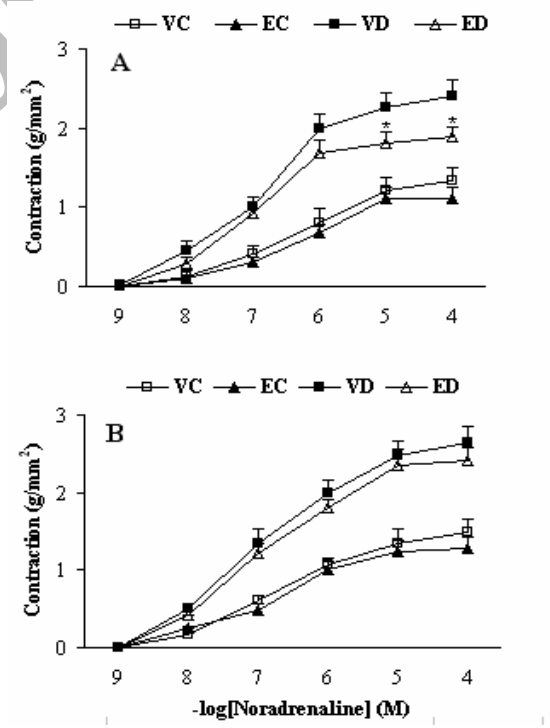


Figure 2. Cumulative concentration-response curves for NA in aortic preparations one month after the experiment in the presence (A) or absence (B) of endothelium. Contractile responses are expressed as grams of tension per cross sectional area (mm^2). Data are shown as means ± SEM.

* P<0.05, ** P<0.01 (Compared to VD)(VC, EC, VD, and ED represent vehicle-treated control, extract-treated control, vehicle-treated diabetic, and extract-treated diabetic rats at a dose of 200 mg/kg of the extract).

Table 1. Body weight and serum glucose levels of control, diabetic, and TFG extract-treated diabetic rats

	Body weight (g)		Serum glucose (mg/dl)	
	Before	After	Before	After
Control*	268.3 ± 4.9*	279.6 ± 5.5	138.7 ± 4.8**	141.3 ± 4.6*
Control + TFG	271.6 ± 4.8*	254.3 ± 5.1	136.2 ± 5.4*	117.2 ± 5.9*
Diabetic*	274.3 ± 6.4*	219.8 ± 4.9**	142.3 ± 4.9*	389.1 ± 13.7***
Diabetic + TFG	256.5 ± 5.2*	237.2 ± 5.2	151.7 ± 4.1	243.2 ± 10.6**#

* P<0.05, ** P<0.005, *** P<0.001 (In comparison with control); # p<0.01 (In comparison with diabetic)

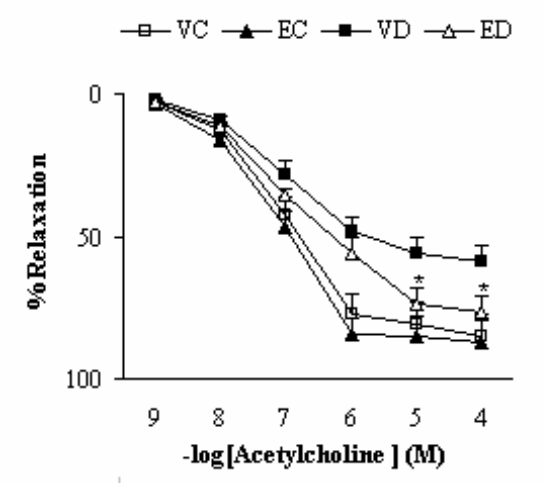


Figure 3. Cumulative concentration-response curves for ACh in NA-precontracted aortic preparations, one month after the experiment. Relaxation response is expressed as percentage. Data shown as means ± SEM. * P<0.05 (Compared to VD)(VC, EC, VD, and ED represent vehicle-treated control, extract-treated control, vehicle-treated diabetic, and extract-treated diabetic rats at a dose of 200 mg/kg of the extract).

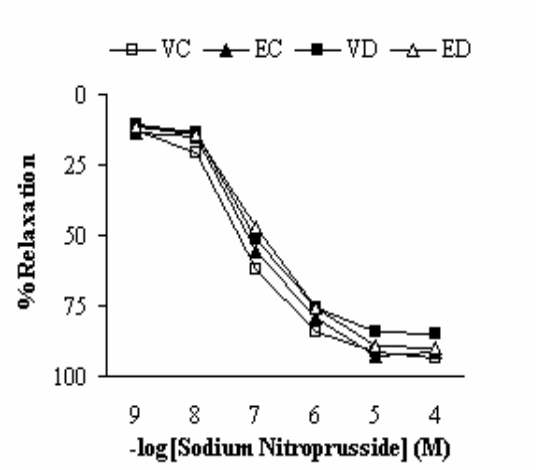


Figure 4. Cumulative concentration-response curves for SNP in NA-precontracted endothelium denuded aortic preparations one month after the experiment. Relaxation response is expressed as percentage. Data are shown as means. (VC, EC, VD, and ED represent vehicle-treated control, extract-treated control, vehicle-treated diabetic, and extract-treated diabetic rats at a dose of 200 mg/kg of the extract).

The contractile responses to NA at concentrations higher than 10^{-7} M in vehicle-treated diabetic rats were found to be significantly higher than vehicle-treated control rats both in the presence (Fig. 2A) or absence of endothelium (Fig. 2B), and treatment of diabetic rats with TFG caused a significant reduction in contractile response to NA only in endothelium-intact rings. Furthermore, treatment of control rats with TFG extract did not produce any significant change in response to KCl and NA in both endothelium-intact and-denuded aortic rings.

Addition of ACh resulted in concentration-dependent relaxations in all aortic rings precontracted with NA (Fig. 3). As it was expected, endothelium-dependent relaxation responses induced by ACh was significantly lower in vehicle-treated diabetic rats compared to the vehicle-treated controls ($p<0.01$). Meanwhile, differences between extract-treated and vehicle-treated diabetic rats were only significant ($p<0.05$) at concentrations higher than 10-5 M. However, relaxation response of extract-treated control was not significantly different from the control group. The endothelium-independent relaxation responses for SNP were also found not to be significantly different amongst groups (Fig. 4), indicating the importance of the presence of endothelium for the beneficial effects of this extract.

Pre-incubation of aortic rings with L-NAME almost completely abolished the vasodilatation response to ACh in segments from extract-treated control and diabetic rats (Fig. 5). Meanwhile, differences between control and extract-treated control and between diabetic and extract-treated diabetic groups were not statistically significant, indicating that the effect of TFG extract on aorta is not due to induction of basal NO release. On the other hand, pre-incubation of aortic segments from extract-treated control and diabetic rats with INDO diminished the endothelial vasodilatation response to acetylcholine partially (Fig. 6). In this respect, differences between control and extract-treated control and between diabetic and extract-treated diabetic groups were statistically significant ($p<0.01$), indicating that the effect of TFG extract on aorta is partly due to enhanced basal activity of cyclooxygenase and related prostanoid biosynthetic pathway by a predominance of vasorelaxant factors.

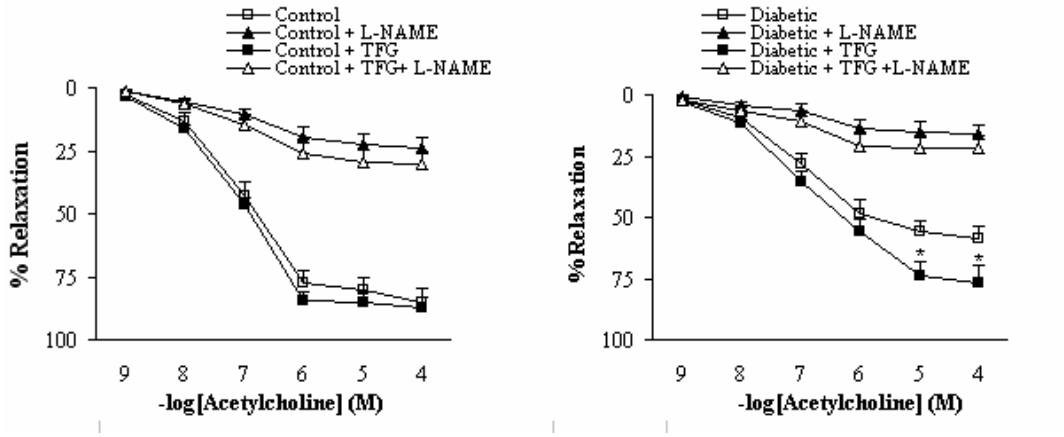
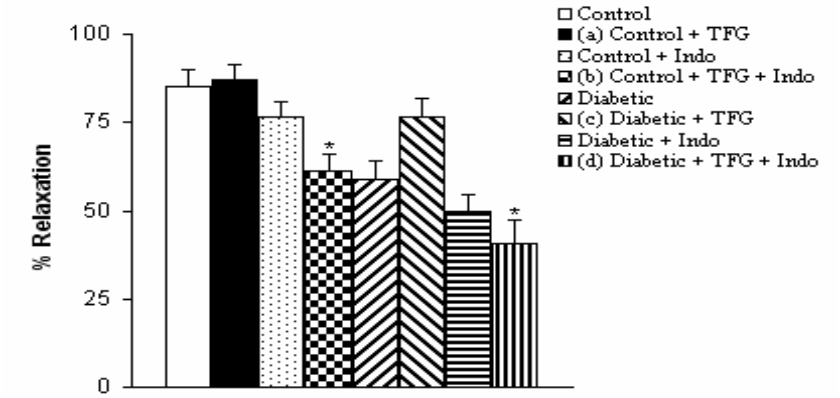


Figure 5. Cumulative concentration-response curves for ACh in NA-precontracted aortic preparations one month after the experiment in the absence and presence of L-NAME (at a concentration of 100 μM 30 min before experiment). Relaxation response is expressed as percentage. Data are shown as means ± SEM.
* P<0.05 (Compared to VD)(VC, EC, VD, and ED represent vehicle-treated control, extract-treated control, vehicle-treated diabetic, and extract-treated diabetic rats at a dose of 200 mg/kg of the extract).



Fiure 6. Maximum relaxation response for ACh in NA-precontracted aortic preparations one month after the experiment in the absence and presence of indomethacin (at a concentration of 10 μM 30 min before experiment). Relaxation response is expressed as percentage. Data are shown as means ± SEM.
* P<0.01 (a vs. b or c vs. d)(VC, EC, VD, and ED represent vehicle-treated control, extract-treated control, vehicle-treated diabetic, and extract-treated diabeti

DISCUSSION

The results of the present study demonstrated that aorta of one-month STZ-diabetic rats are more responsive to the contractile effect of NA as an α-adrenoceptor agonist and KCl as a non-specific agent both in the presence and absence of endothelium than those of the corresponding control groups. Similar results showing the increased vascular responsiveness to contractile agents in STZ-diabetic rats have been reported in previous studies (10-11). This increased vascular smooth muscle responsiveness in diabetic rats could be attributed to deficient endothelial activity (10-11), enhanced phosphoinositide (PI) metabolism (12), enhanced sensitivity of calcium channels (13), and increased sensitivity to adrenergic agonists (13). Furthermore, oxidative stress is increased due to excessive production of oxygen-free radicals and decreased antioxidant defense systems (14-16).

In this study, alcoholic seed extract of fenugreek attenuated the increased responsiveness of aortic rings in diabetic state. Our results demonstrated that fenugreek extract at a dose of 200 mg/kg could partially counteract the increased contractile response of endothelium-intact aortic rings of diabetic rats following NA and/or KCl and could enhance the relaxation response to ACh in diabetic rats. The beneficial effects of chronic fenugreek extract treatment on NA and KCl induced contractions was specific for aortas of diabetic rats, because the extract treatment did not produce any significant changes in control preparations.

The results of this study also showed that the beneficial effects of TFG extract in aorta of

diabetic rats is partly mediated through pathway of prostaglandin biosynthesis and there is not significant changes in the basal capacity of endothelium for the release of NO. As it has been indicated by the results of other studies, in the presence of indomethacin, increase in the release of the vasoconstrictor prostanoids and decrease in release of vasorelaxant prostanoids are important mechanisms in the endothelial dysfunction induced by diabetes as well as abnormality associated with different cardiovascular risk factors (17-18).

In conclusion, on the basis of the findings of this study, chronic treatment of diabetic rats with alcoholic seed extract of fenugreek partially prevented development of changes in vascular reactivity and endothelial prostaglandins are essential for these effects.

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