

Soybean feeding improves vascular dysfunction and attenuates oxidative stress in streptozotocin-diabetic rats

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Background and Objective: The effect of chronic dietary soybean (*Glycine max L.*) on aortic reactivity of streptozotocin (STZ)-diabetic rats was studied. **Materials and Methods:** Male diabetic rats received soybean for 7 weeks 1 week after diabetes induction at weight ratios of 3 and 6%. Contractile responses to KCl and phenylephrine (PE) and relaxation response to acetylcholine (ACh) were obtained from aortic rings.

Results: Maximum contractile response of endothelium-intact rings to PE was significantly lower in soybean6%-treated diabetic rats relative to untreated diabetics ($p < 0.05$) and endothelium removal abolished this difference. Endothelium-dependent relaxation to ACh was significantly higher in soybean6%-treated diabetic rats as compared to diabetic ones ($p < 0.05$) and pretreatment of rings with nitric oxide synthase inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) and/or indomethacin attenuated it. Two-month diabetes also resulted in an elevation of malondialdehyde (MDA) and decreased superoxide dismutase (SOD) activity and soybean treatment significantly reversed the increased MDA content and reduced activity of SOD.

Conclusion: Therefore, chronic treatment of diabetic rats with soybean could dose-dependently prevent some abnormal changes in vascular reactivity in diabetic rats through nitric oxide- and prostaglandin-related pathways and via attenuation of oxidative stress in aortic tissue and endothelium integrity seems essential for this effect.

1. Introduction

The prevalence of diabetes mellitus (DM) is increasing worldwide and it is a major health problem in the 21st century and prevalence of DM is estimated to increase to 366 million by 2030 (1). Cardiovascular disorders

continue to constitute major causes of morbidity and mortality in diabetic patients in spite of significant achievements in their diagnosis and treatment (2). Changes in vascular responsiveness to vasoconstrictors and vasodilators are mainly responsible for development of some vascular complications in diabetes (3). Most of

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these complications are due to increased serum glucose and augmented generation of reactive oxygen species (ROS) which finally lead to endothelium dysfunction (4).

Soybean (*Glycine max* L.) has long been known as one important source of protein. In addition to protein, soybean also contains various nutrients and functional components including isoflavones with many potential benefits (5). Epidemiological evidence suggests that consuming soy products in population lowers incidence of cardiovascular disease and this has led to the suggestion that soybean and its isoflavones may be beneficial for cardiovascular health due to their protective property (6-8). Studies in experimental animals in which cardiovascular responses to soybean and its constituents have been assessed are broadly supportive of its protective role. In this respect, soybean has been shown to improve vascular function through nitric oxide pathway (9, 10). Endothelium-dependent relaxation of rat aorta and main pulmonary artery by soybean and its effective substances has also been reported (11). Meanwhile, it has been reported that soybean supplementation would be helpful to control blood glucose and serum lipid in diabetic patients and soybean exhibits an antioxidant activity that may contribute to enhance the effect of antioxidant defense and this activity contributes to protection against oxidative damage in patients with diabetes (12). Even, it has been claimed that soybean and its constituents may improve glucose homeostasis and delay progression of type 2 diabetes as reviewed before (13). Nevertheless, the *in vivo* protective effect of soybean on vascular system in diabetes has not been documented yet. Therefore, this study was designed to assess for the first time the beneficial effect of chronic dietary soybean 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 (Pasteur's institute, Tehran, Iran) weighing 220-280 g were housed in an air-conditioned colony room at 21 ± 2 °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.

2.2. Experimental protocol

The rats ($n = 48$) 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 six groups (eight in each): normal control, soybean-treated controls in two subgroups, diabetic, and soybean-treated diabetics in two subgroups. Standardized soybean powder was mixed with standard rat chow at weight ratios of 3 and 6% and was feed to animals throughout the experimental period for 7 weeks. These ratios were determined according to our pilot study and earlier reports on the beneficial effect of soybean. Changes in body weight were regularly recorded during the study.

Finally, the rats were 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% CO₂ and 95% O₂. 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 μ m) and once the contraction stabilized, a single concentration of acetylcholine (1 μ 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)-induced vasodilatation in rings with endothelium, they were precontracted with a submaximal concentration of PE (10⁻⁶ M) which produced 70-80% of maximal response. The sensitivity to the agonists was evaluated as pD₂, which is the negative logarithm of the concentration of the drug required to produce 50% of the maximum response.

To determine the participation of NO, rings were incubated 30 min before the experiment with L-NAME (100 μ M, a non-selective NOS inhibitor). To determine the participation of endothelial vasodilator factors in response to ACh, segments were incubated with INDO (10 μ M, an inhibitor of COX-derived prostanoid synthesis) 30 min before the experiment with ACh.

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 (14).

2.3. Determination of MDA concentration in aortic rings

After removing aortic segments and cleansing

them of extra tissues, they were blotted dry and weighed, then made into 5% tissue homogenate in ice-cold 0.9% saline solution. A supernatant was obtained from tissue homogenate by centrifugation (1000 \times g, 4 $^{\circ}$ C, 5 min). The MDA concentration (thiobarbituric acid reactive substances, TBARS) in the supernatant was measured as described before (15). Briefly, trichloroacetic acid and TBARS reagent were added to supernatant, then mixed and incubated at 100 $^{\circ}$ C for 80 min. After cooling on ice, samples were centrifuged at 1000 \times g for 20 min and the absorbance of the supernatant was read at 532 nm. TBARS results were expressed as MDA equivalents using tetraethoxypropane as standard.

2.4. Measurement of SOD activity in aortic rings

The supernatant of tissue homogenate were obtained as described earlier (16). Briefly, supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37 $^{\circ}$ C) for 40 min and NBT was added. Blue formazan was then monitored spectrophotometrically at 550 nm. The amount of protein that inhibited NBT reduction to 50% maximum was defined as 1 nitrite unit (NU) of SOD activity.

2.5. Drugs

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

2.6. 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 diabetic rats was found to be significantly decreased as compared to controls ($p < 0.01$) and soybean treatment at both ratios, especially at a ratio of 6%, caused a significant lower reduction in weight in diabetic rats as compared to 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 soybean at a ratio of 6% caused a significant decrease in the serum glucose level relative to diabetics ($p < 0.05$). In addition, soybean treatment of control rats did not produce any significant change regarding serum glucose level (Fig.1).

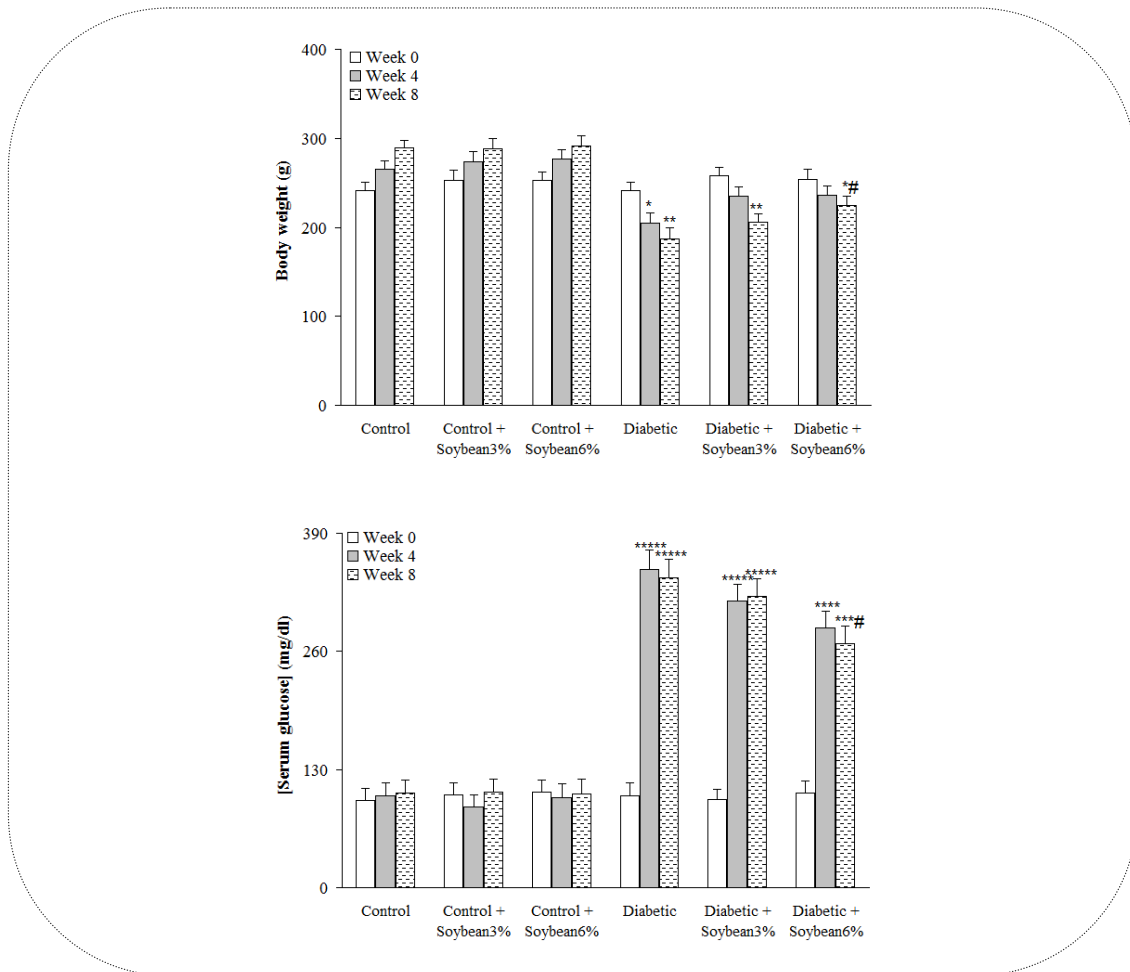


Fig. 1: Body weight and serum glucose concentration in different weeks (means \pm S.E.M). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, ***** $p < 0.0005$ (as compared to week 0 in the same group) # $p < 0.05$ (relative to diabetic group in the same week)

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 treated diabetic rats in the presence of endothelium were found to be significantly ($p < 0.01-0.005$) greater

than control rats and concentration-response curve of endothelium-intact aortas from soybean-treated diabetic rats (at a ratio of 6%) to KCl and PE was significantly attenuated compared to 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 soybean. In addition, aortic rings with endothelium from soybean-treated control group showed a non-significant reduction in contractile response to KCl and PE as compared to controls. There were also no significant differences among the groups in terms of the pD₂ (data not shown), indicating that there has not been any significant change in the sensitivity of aortic rings from different groups.

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 treated diabetic rats in relation to treated controls ($p < 0.05-0.005$). Meanwhile, the existing difference between soybean-treated (at a ratio of 6%) and diabetic rats was only significant ($p < 0.05$) at concentrations higher than 10⁻⁵ M. Relaxation

response of soybean-treated control rats was non-significantly greater than control group.

Regarding relaxation response to ACh, pre-incubation of aortic rings with L-NAME almost completely abolished the vasodilator response to ACh in segments from soybean6%-treated diabetic rats, indicating the important role of endothelium-derived NO in the vascular effect of soybean (Fig. 5). Pre-incubation of aortic segments from soybean6%-treated diabetic rats with INDO also moderately and significantly diminished the endothelial vasodilator response to ACh ($p < 0.05$) (Fig. 6).

Regarding aortic lipid peroxidation markers (Table1), STZ-induced diabetes resulted in an elevation of MDA content and decreased SOD activity ($p < 0.005-0.001$) in aortic tissue and chronic treatment of diabetic group with soybean at a ratio of 6% significantly reversed the increased MDA content ($p < 0.05$) and reduced activity of SOD ($p < 0.005$).

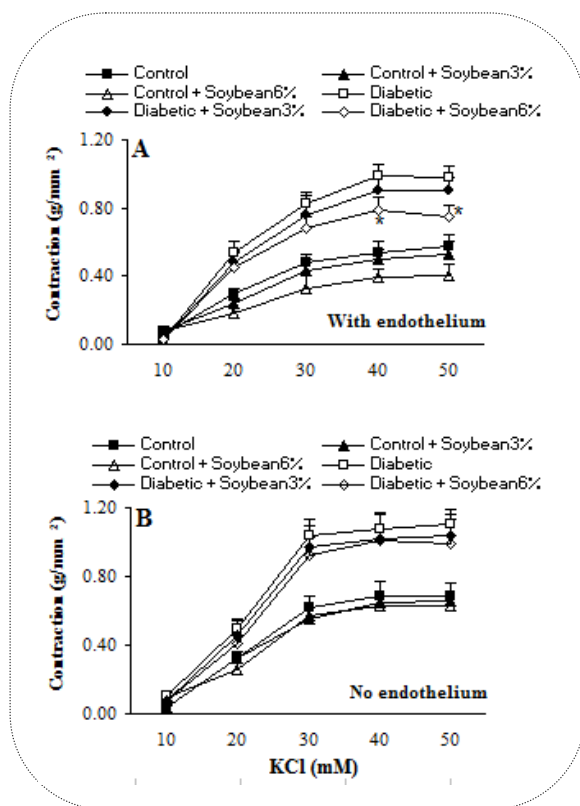


Fig. 2: Cumulative concentration-response curves for KCl in aortic preparations 8 weeks after experiment in the presence (A) and absence (B) of endothelium (means \pm S.E.M). * $p < 0.05$ (as compared to diabetic)

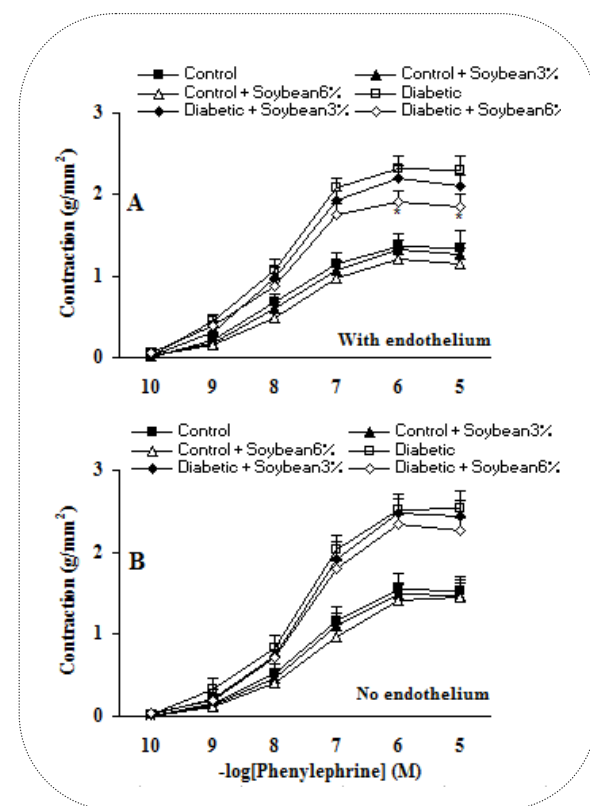


Fig. 3: Cumulative concentration-response curves for PE in aortic preparations 8 weeks after experiment in the presence (A) and absence (B) of endothelium (means \pm S.E.M). * $p < 0.05$ (as compared to diabetic)

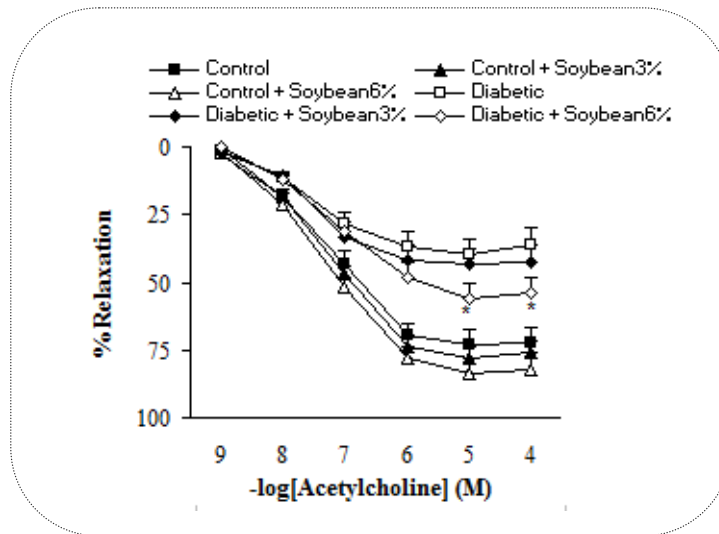


Fig. 4: Cumulative concentration-response curves for ACh in endothelium-intact aortic rings precontracted with PE 8 weeks after experiment. Relaxation responses are expressed as a percentage of the submaximal contraction induced by phenylephrine which produced 70-80% of maximal response (means \pm SEM). * $p < 0.05$ (as compared to diabetic)

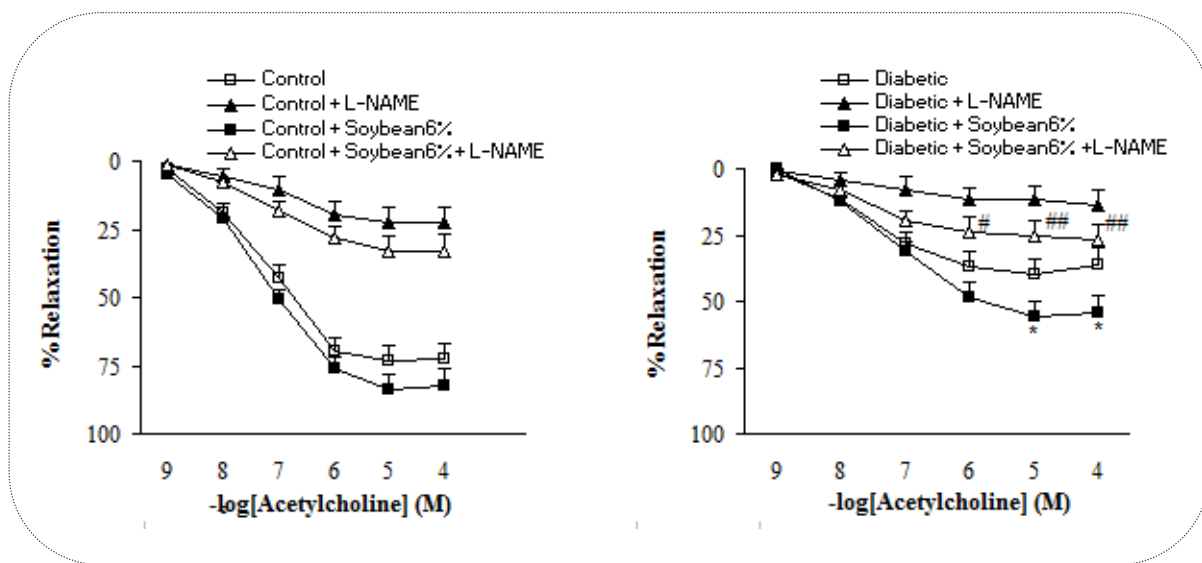


Fig. 5: Cumulative concentration-response curves for ACh in endothelium-intact aortic rings precontracted with phenylephrine in the presence and absence of L-NAME 8 weeks after the experiment in control and diabetic rats. Relaxation responses are expressed as a percentage of the submaximal contraction induced by phenylephrine which produced 70-80% of maximal response (means \pm SEM). L-NAME stands for N(omega)-L-arginine methyl ester. * $p < 0.05$ (as compared to diabetic), # $p < 0.05$, ## $p < 0.01$ (as compared to diabetic+soybean6%).

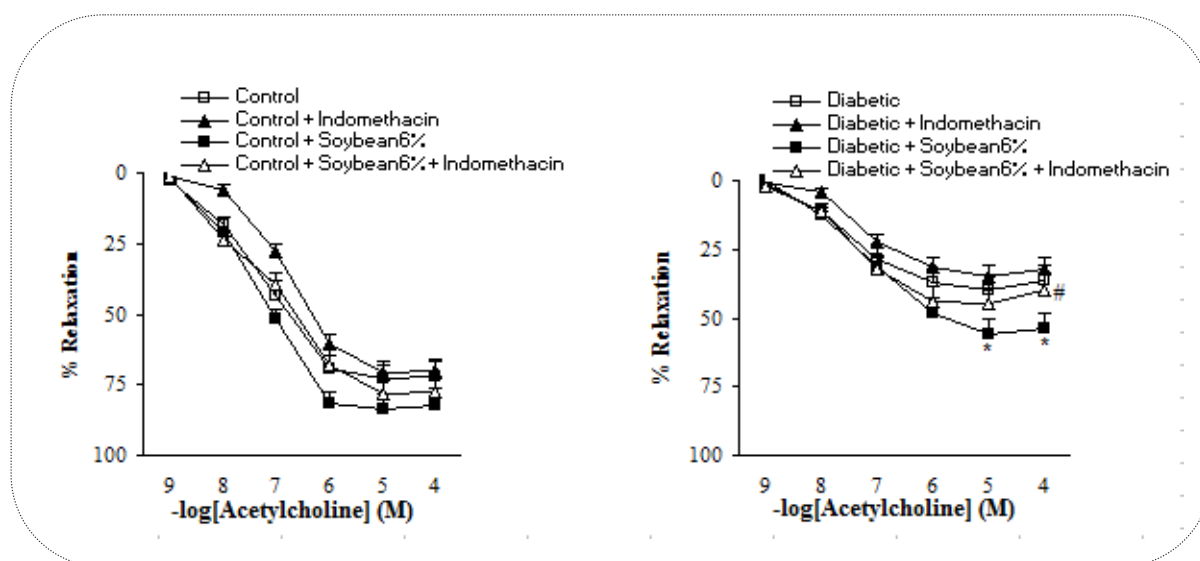


Fig. 6: Maximum relaxation response to acetylcholine (ACh) in aortic rings precontracted with phenylephrine in the presence and absence of indomethacin eight weeks after experiment in control and diabetic rats. Relaxation responses are expressed as a percentage (means \pm SEM). * $p < 0.05$ (as compared to diabetic) # $p < 0.05$ (as compared to diabetic+soybean6%)

Table 1: Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity in aortic tissue of studied groups

Groups	MDA (μ mol.g-1 protein)	SOD activity (kNU.g-1 protein)
Control (n=7)	5.7 \pm 0.5	117 \pm 6
Control + Soybean6% (n=6)	5.4 \pm 0.7	119 \pm 7
Diabetic (n=6)	9.2 \pm 0.7*	76 \pm 8**
Diabetic + Soybean6% (n=7)	7.1 \pm 0.8#	91 \pm 7*##

* $p < 0.005$, ** $p < 0.001$ (vs. control group); # $p < 0.05$, ## $p < 0.005$ (vs. diabetic group)

4. Discussion

In this study, administration of soybean for 7 weeks did have a mild hypoglycemic effect, it reduced the enhanced contractility of aortic rings to KCl and PE and increased ACh-induced relaxation which was partly due to involvement of NO and prostaglandins pathways since the relaxation was blocked in the presence of L-NAME and/or INDO. In addition, endothelium removal clearly affected PE-induced contractions in soybean-treated diabetic rats. Regarding oxidative stress markers, soybean treatment attenuated the increased MDA content and reduced activity of SOD.

Vascular dysfunction is one of the complicating features of diabetes in humans and its experimen-

tal model and hyperglycemia is the primary cause of micro and macrovascular complications in diabetic condition (17). 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 (15) and chronic soybean was capable to attenuate this change only for PE-induced contractions. Impaired endothelial function (18), enhanced sensitivity of calcium channels (19), an increase in vasoconstrictor prostanoids due to increased superoxide anions and increased sensitivity to adrenergic agonists (20) might all be responsible for increased contractile responses in diabetic rats, which may have been improved following soybean 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 (21-23). The ACh-induced relaxation response is endothelium-dependent and NO-mediated (15). 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 soybean treatment. Although some researchers asserted that the sensitivity to acetylcholine decreases in diabetes (20), the results of this research, in accordance with those of many previous ones (24) 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 mechanisms, including advanced glycation end product (AGE) formation, increased polyol pathway flux, apoptosis and reactive oxygen species (ROS) formation (25). Our results showed that soybean treatment could dose dependently exert a mild hypoglycemic effect in STZ-induced diabetic rats, therefore, its beneficial effect on aortic tissue of diabetic rats should be partly 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 (16) and as was observed in this study. This could also lead to diabetes-induced functional changes in vascular endothelial cells and the development of altered endothelium-dependent vasoreactivity. The results of the present study showed that chronic treatment of soybean significantly decreased MDA content and enhanced SOD activity in aortic tissue from diabetic rats, indicating that the improvement in vascular responsiveness from soybean may be partly due to ameliorating lipid peroxidation and oxidative injury. These results clearly suggested that another cause of the effect of soybean on improving the endothelial dysfunction is due to its antioxidative activity.

In conclusion, to the best of our knowledge, this is the first study to report that in vivo chronic treatment of diabetic rats with soybean dose-dependently could prevent the functional changes in vascular reactivity observed in diabetic rats through nitric oxide- and prostaglandin-dependent pathways and via attenuation of aortic lipid peroxidation. Our data may be helpful in the development of new natural drugs to improve endothelial function and to prevent cardiovascular diseases.

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