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Exercise Training and Grape Seed Extract Co-administration Improve Endothelial Dysfunction of Mesenteric Vascular Bed in STZ-induced Diabetic Rats

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Abstract: Endothelial dysfunction represents a hallmark of diabetic vascular complications. Although, regular exercise training or antioxidants could prevent these complications to some extent, the effects of grape seed extract as an antioxidant alone or combined with exercise on the diabetic induced endothelial dysfunction was not investigated. Therefore, the aim of this study was to determine the combined effect of grape seed extract and exercise training on vascular endothelial function in streptozotocin induced diabetic rats. Forty five male Wistar rats were randomly divided into five groups: Sedentary control, sedentary diabetic, trained diabetic and sedentary or trained diabetic that received 200 mg kg^{-1} grape seed extract. Eight weeks after diabetes induction by streptozotocin (60 mg kg^{-1}) the body weight and blood glucose were measured and mesenteric vascular bed responses to vasoactive agents (acetylcholine, phenylephrine and sodium nitroprusside) were determined. The data have shown that the weight gain, plasma antioxidant capacity and endothelium dependent vasorelaxation to acetylcholine reduced significantly in diabetic animals. However, exercise training combined with grape seed extract improve body weight gain, increase plasma antioxidant capacity, decrease blood glucose and restores vasodilatory response to acetylcholine more significant than exercise training or grape seed extract alone. On the other hand, the vasoconstrictive response to phenylephrine and vasodilatory response to sodium nitroprusside did not change significantly. The data indicated that exercise training and grape seed extract combination had more significant improving effects on endothelial dysfunction than exercise training or grape seed extract alone and may constitute convenient and inexpensive therapeutic approach to diabetic vascular complications.

Key words: Diabetic vascular complication, plasma antioxidant capacity, exercise, grape seed extract, mesenteric vascular bed

INTRODUCTION

Endothelial damage, caused by oxidative stress and vascular inflammation, represents a hallmark of diabetes and its vascular complications (Watala *et al*., 2009). The initial step in Cardiovascular Diseases (CVD) is endothelial dysfunction, which exposes these cells and the underlying cell layers to a deleterious inflammatory process which finally leads to the formation of atherosclerotic lesions (Fearon and Faux, 2009). In addition, some agents such as statins and angiotensin converting enzyme-inhibitors that improve endothelial function also have antidiabetic activity and inhibit the progression of vascular complications associated with diabetes (Watala *et al*., 2009).

On the other hand, it has been shown that exercise induces angiogenesis and alters vascular reactivity in different vascular beds and decreased blood pressure (Grijalva *et al*., 2008). It has also indicated that both indices of endothelial damage, i.e., increased leukocyte adhesion and impaired endothelium-dependent relaxation was improved by regular low intensity exercise training through amelioration of oxidant/antioxidant levels (Chakraphan *et al*., 2005). These results indicated that regular Exercise Training (ET) could be a non-pharmacological treatment in preventing diabetic cardiovascular problems enhanced by endothelial damage.

Both experimental and clinical data suggest that using antioxidants such as vitamin E in insulin resistance or diabetic states normalizes oxidative stress and improve both endothelium-dependent vasodilation and insulin sensitivity (Laight *et al*., 2000). In addition, it has been also shown that eight weeks treatment with alpha-lipoic acid (Cameron *et al*., 2001) or 2 weeks of high-dose allopurinol (xanthine oxidase inhibitor) treatment

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(Inkster *et al*., 2007) reverses endothelial dysfunction in streptozotocin (STZ)-induced diabetic rats.

The composition and properties of Grape Seed Extract (GSE) have been extensively investigated and reported to have many beneficial effects on human health such as reduction of CVD (Leifert and Abeywardena, 2008; Shao *et al*., 2009) and inhibition of lead induced hypertension in rats (Badavi *et al*., 2008). However, the potential effect of GSE alone or in combination with exercise on the diabetic induced endothelial dysfunction was not investigated. Therefore, GSE could play a favorable role in scavengering free radicals and could thereby ameliorate endothelial dysfunction and potentiate the beneficial effects of ET on endothelial function. Therefore, the aim of this study was to determine the effect of GSE alone or in combination with ET on vascular endothelial dysfunction in STZ-induced diabetic rats.

MATERIALS AND METHODS

Animals: The experiments were conducted from November 2010 to March 2011 on forty-five male Wistar rats weighing 200-232 g obtained from animal house of Ahwaz Jundishapour University of Medical Sciences, Ahwaz, Iran. The animals were randomly divided into five groups: Sedentary Control (SC), Sedentary Diabetic for 8 weeks (SD), Trained Diabetic for 8 weeks (TrD), sedentary diabetic that received GSE for 8 weeks (ExD), trained diabetic that received GSE for 8 weeks (TrExD). Diabetes was induced by a single intraperitoneal injection of streptozotocin (60 mg kg^{-1} body weight) dissolved in normal saline (Cotter *et al*., 2002). The GSE was dissolved in 1 mL distilled water and administered orally via gavage (200 mg kg^{-1}, once a day). Control animals were injected with an equivalent volume of vehicle. All groups were maintained under the same conditions (temperature-controlled room, 22±2°C, with a 12 h dark-light cycle) supplied with food and water ad libitum. Rats were considered diabetic when blood glucose levels were >300 mg d^{-1} (16.7 mmol L^{-1}) five days after STZ injection (Li *et al*., 2008). The protocol and procedures of this study were approved by the Animal Care and Use Committee of the University.

Drugs: Streptozotocin (STZ), acetylcholine chloride, phenylephrine hydrochloride, sodium nitroprusside and heparin sodium were purchased from Sigma (St. Louis, Mo). Sodium chloride, potassium chloride, magnesium sulphate, sodium hydrogen carbonate, potassium hydrogen orthophosphate, D-glucose and calcium chloride were obtained from Merck Laboratories, Ketamine and Xylazine from Alfasan Co (Woderen-Holland).

Exercise training protocol: Rats conducted exercise training on treadmill daily for 8 weeks, 1 day after diabetic verification as shown in Table 1 after gavage of GSE.

Preparation of grape seed extract: Grape seeds (*Vitis vinifera* L.) was separated from the grapes manually, dried in shade (25-30°C) for a week and then fine powdered using a mill. The grape seed powder was macerated in 70% ethanol (25% w/v) for 3 days at room temperature and was stirred 3 times daily. The mixture was filtered with few layers of cheese cloth and the filtrate was dried at room temperature (25-30°C) to evaporate ethanol and the GSE was obtained (about 25%) as a powder (Badavi *et al*., 2008).

Isolated perfused mesenteric bed: The animals were anesthetized by Ketamine 80 mg kg^{-1}+Xylazine 12 mg kg^{-1} containing 1000 U kg^{-1} heparin, the abdominal cavity was opened by a mid-line incision through the linea alba and the mesenteric bed was excised using the procedure described by McGregor (1965). A PE 50 tube inserted into the superior mesenteric artery and then perfused with Krebs solution at a constant rate (5 mL min^{-1}) via peristaltic pump (Gilson-France). The isolated, perfused preparations were placed in water-jacketed chamber at 37°C. The tissue was allowed to equilibrate for 30 min before commencing the experiments. The pressor response to different concentrations of phenylephrine (1-100 μM) was recorded using a pressure transducer (Powerlab, ADInstrument, Australia). The vasoconstriction was expressed as a percent of increase in perfusion pressure in response to phenylephrine. In pre-contracted mesenteric bed (with 10 μM phenylephrine), the vasodilator response to acetylcholine (endothelium-dependent vasodilator, 0.01-100 μM) and sodium nitroprusside (endothelium independent vasodilator, 0.001-0.1 μM) were measured. The responses were expressed as percent of relaxation of the phenylephrine-induced precontraction.

Table 1: Exercise training protocol for rats on treadmill

Week	Belt speed (m min^{-1})	Inclination (°)	Total time (min)
1	16	0	30
2	16	5	30
3	16	10	45
4	16	12	45
5	16	12	60
6	16	12	60
7	16	12	60
8	16	12	60

Total plasma antioxidant capacity: Total antioxidant status is a marker of oxidative stress, measured as the total radical scavenging capacity of the plasma. It was evaluated using FRAP (ferric reducing ability of plasma) assay (Benzie and Strain, 1996). The method measures the ability of antioxidants contained in the sample to reduce ferric-2, 4, 6-tripyridyl-s-triazine (Fe^{3+}-TPTZ) to ferrous (Fe^{2+}) form, which is blue-colored and absorbs light at 593 nm. The change in absorbance is converted into a FRAP value (in μM) by relating the change of absorbance at 593 nm of test sample to that of standard solutions of known FRAP values (10-1000 μmol L^{-1}).

Statistical analysis: Results were analyzed using SPSS version 16 and expressed as Mean ± S.E.M. and comparisons between groups in each protocol were performed using repeated measurement ANOVA followed by LSD multiple comparison test or student t-tests as appropriate. The p-values of less than 0.05 were considered significant.

RESULTS

Body weight and blood glucose levels: At the beginning of experiment, the body weight of animals in all groups was not different from each other, but 8 weeks after STZ administration, weight gain of diabetic groups was significantly less than control group (diabetic: 234.9±7.4 g vs. control: 301.9±5.3 g, $p<0.001$, one way ANOVA followed by LSD, Table 2). However, the final weight of TrExD group was significantly more than other diabetic groups, although did not reach that of control group (Table 2).

Regarding blood glucose, ET and GSE alone or in combination significantly reduced the blood glucose elevation in STZ induced diabetic animals (TrD: 463.0±34.4; ExD: 470.7±28.6 and TrExD: 406.6±42.7) compared to sedentary diabetic group (SD: 567.1±16.4). Nevertheless, co-administration of ET and GSE reduced blood glucose elevation in diabetic animals (TrExD: 406.6±42.7) more significantly than ET or GSE alone, but this values are so far from corresponding control values (Table 2).

Basal perfusion pressure in the isolated mesenteric bed: Basal perfusion pressure of mesenteric vascular bed isolated from Sedentary Diabetic (SD) group was not statistically different from those of SC, TrD, ExD and TrExD groups (56.9±8.2, 47.1±4.4, 42.7±1.5, 45.9±5.5 and 43.7±2.8 mmHg, respectively Fig. 1).

Vasodilator responses to acetylcholine and sodium nitroprusside: Endothelial dependent vasodilator response to different doses of acetylcholine (0.01-100 μM) was reduced significantly in the SD group (Fig. 2). Furthermore, ET or GSE administration alone partially improved the vasodilator response to acetylcholine in the STZ induced diabetic animals, so that the vasodilator response of TrD and ExD groups were significantly more than SD group (SD: 34.2%±3, vs. TrD: 50.1%±3.9 and ExD: 48.1%±3.3 for Ach 100 μM). However, combination of exercise and GSE, improved the vasodilator responses more significantly, so that there was no significant difference between SC and TrExD groups in response to different doses of Ach (SC: 70.9%±3.6 vs. TrExD: 65.7%±4.8 for Ach 100 μM, Fig. 2). However, the endothelium independent vasorelaxation to sodium nitroprusside (0.001-0.1 μM) was not changed in the phenylephrine (10 μM) pre-contracted mesenteric bed in different groups (71.7%±4.4, 66.8%±7.5, 74.9%±5.7, 58.8%±6.1 and 77.1%±5.3 in SC, SD, TrD, ExD and TrExD groups, respectively Fig. 3).

Table 2: Body weight and blood glucose (Mean±SEM, n = 10-15) before and 8 weeks after STZ or vehicle administration in Sedentary Control (SC), Sedentary Diabetic (SD), Trained Diabetic (TrD), Extract Diabetic (ExD) and trained+extract diabetic (TrExD) groups

Groups	Pre-STZ weight (g)	Final weight (g)	Final Blood glucose (mg dL^{-1})
SC	209.6±3.8	301.9±5.3 ***	100.7±4.6
SD	216.8±3.1	234.9±7.4 * , ‡‡‡	567.1±16.4 ‡
TrD	217.7±3.6	214.6±9.7 ‡‡‡	463.0±34.4 ‡,¶
ExD	208.7±2.3	222.6±6.6 ‡‡‡	470.7±28.6 ‡,¶
TrExD	214.5±2.9	260.0±9.6***,‡‡‡,¶	406.6±42.7 ‡,¶

*p<0.05, ***p<0.001, significantly different from corresponding pre-STZ value (paired t-test). ‡ p<0.05, ‡‡‡ p<0.001, significantly different from corresponding value in control group (one way ANOVA followed by LSD) ¶ p<0.05, significantly different from corresponding value in sedentary diabetic group (one way ANOVA followed by LSD)

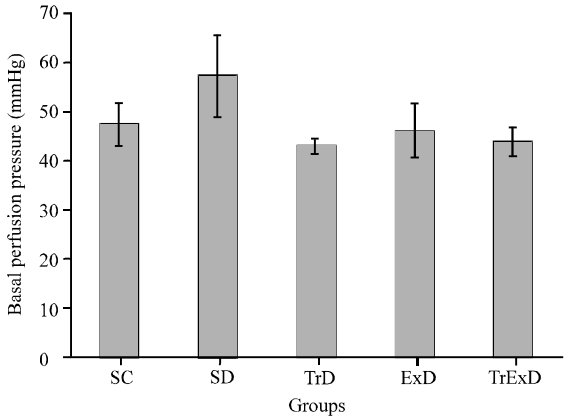

Fig. 1: Basal perfusion pressure (Mean±SEM, n = 8-10) of isolated mesenteric vascular bed isolated from Sedentary Control (SC), Sedentary Diabetic (SD), exercise Trained Diabetic (TrD), GSE Treated Diabetic (ExD) and exercise trained + GSE treated diabetic (TrExD) groups. There was no significant difference between groups

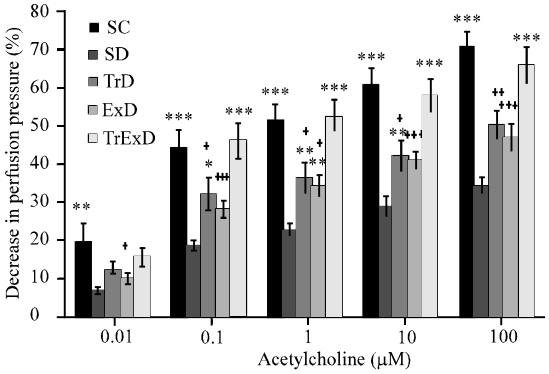

Fig. 2: Vasodilator responses to acetylcholine (Mean±SEM, n = 8-10) in the phenylephrine (10 μM) pre-contracted mesenteric vascular beds, isolated from Sedentary Control (SC), Sedentary Diabetic (SD), Exercise Trained Diabetic (TrD), GSE Treated Diabetic (ExD) and exercise trained+GSE treated diabetic (TrExD) groups. (*p<0.05, **p<0.01, ***p<0.001) significantly different from sedentary diabetic group, (†p<0.05, ††p<0.01, †††p<0.001) significantly different from control group (tow way ANOVA followed by LSD)

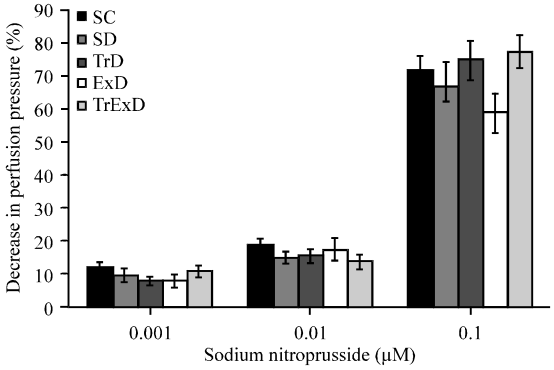

Fig. 3: Vasodilator responses to sodium nitroprusside (Mean±SEM, n = 8-10) in the phenylephrine (10 μM) pre-contracted mesenteric vascular beds, isolated from Sedentary Control (SC), Sedentary Diabetic (SD), Exercise Trained Diabetic (TrD), GSE treated diabetic (ExD) and exercise trained+GSE treated diabetic (TrExD) groups. There was no significant difference between groups

Vasoconstrictor response to phenylephrine: The Vasoconstrictor response to phenylephrine (1-100 μM) was not different in mesenteric vascular bed isolated from different groups of the experiment (195.7±40.2%, 235.9±38.3%, 199±18.6%, 235±50% and 261.9±36.46% for SC, SD, TrD, ExD and TrExD groups, respectively Fig. 4).

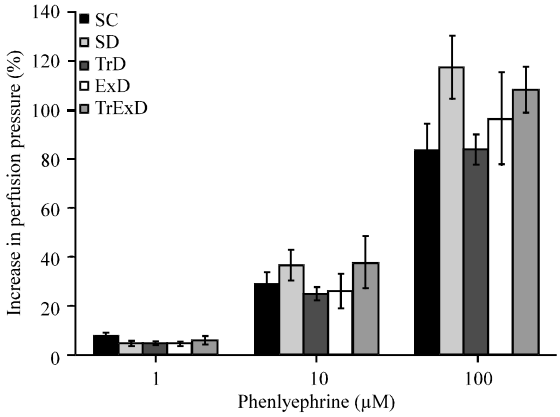

Fig. 4: Vasoconstrictor response (Mean±SEM, n = 8-10) to phenylephrine of isolated mesenteric vascular bed of Sedentary Control (SC), Sedentary Diabetic (SD), Exercise Trained Diabetic (TrD), GSE treated diabetic (ExD) and exercise trained + GSE treated diabetic (TrExD) groups. There was no significant difference between groups.

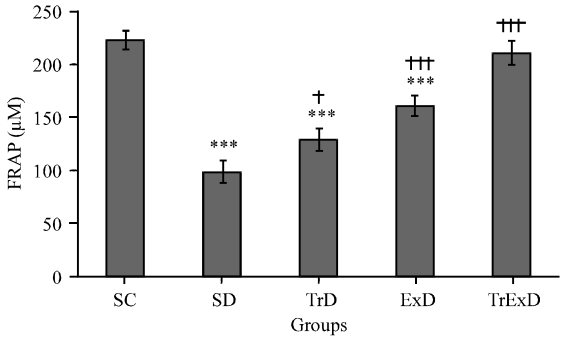

Fig. 5: Total plasma antioxidant capacity measured as ferric reducing ability of plasma (FRAP, Mean±S.E.M, n = 10) of Sedentary Control (SC), Sedentary Diabetic (SD), Exercise Trained Diabetic (TrD), GSE treated diabetic (ExD) and exercise trained+GSE treated diabetic (TrExD) groups. *** p<0.001 different from sedentary control group, †p<0.05, ††p<0.001 different from sedentary diabetic rats; one-way ANOVA followed by LSD test

Total plasma antioxidant capacity: The total antioxidant capacity of plasma was reduced significantly in SD rats (99.3±10.6 μM) compared with SC (224±8.5 μM) and the other groups. However, as shown in Fig. 5 exercise training or GSE administration partially returned the plasma antioxidant capacity toward normal (129.8±10.2 and 161.4±9.2 μM, respectively). Moreover, this increment in plasma antioxidant capacity was more significant when exercise training was combined with grape seed extract

(211.4±12.1 μM, $p<0.001$, one-way ANOVA followed by LSD test, compared with SD group).

DISCUSSION

As expected, the endothelium dependent vasorelaxation to acetylcholine and total antioxidant capacity reduced significantly in diabetic animals and administration of ET or GSE alone improve this change partially. However, combination of ET and GSE, restore plasma antioxidant capacity and endothelial function more completely and reduced blood glucose significantly. On the other hand, the basal perfusion pressure, vascular responses to phenylephrine and sodium nitroprusside did not change significantly.

Supporting of these results, several studies have been shown that endothelium-dependent relaxation has been impaired in blood vessels from spontaneously diabetic (Durante *et al*., 1988) and STZ-induced diabetic rats (Pieper and Gross, 1988). Vascular endothelial dysfunction is characterized by reduced activation of endothelial Nitric Oxide Synthase (eNOS), reduced generation and bioavailability of Nitric Oxide (NO) and increased production of Reactive Oxygen Species (ROS) (Calles-Escandon and Cipolla, 2001; Ulker *et al*., 2003). Diabetes mellitus may affect endothelial function either by hyperglycemia and/or diabetes induced oxidative stress. Furthermore, it has been shown that hyperglycemia stimulates synthesis of diacylglycerol and the subsequent activation of protein kinase C that constitutes an important signaling pathway leading to endothelial dysfunction (Fatehi-Hassanabad *et al*., 2006). As the blood glucose of the TrExD group was reduced, the improvement of endothelial function may be attributed in part to reduction of blood glucose in this group. It was reported that GSE and its ethyl acetate/ethanol fraction reduced blood glucose and glycosylated hemoglobin (HbA1c) level in a model of type 2 diabetic mice (Hwang *et al*., 2009). In addition, it has been shown that ET could improve hyperglycemia and insulin resistance (Minami *et al*., 2002) and decreased glycosylated hemoglobin (HbA1c) (Grijalva *et al*., 2008). Therefore, these studies support that combination of ET and GSE may potentiate their beneficial effects, reduce blood glucose and improve endothelial function more effectively.

In addition, it has been shown that diabetes mellitus is associated with increased oxidative stress (Davi *et al*., 1999; Ceriello *et al*., 2002). ROS may impair endothelial function through inactivation of nitric oxide or reduction of Endothelium-Dependent Hyperpolarizing Factor (EDHF) (Fukao *et al*., 1997) or by serving as an endothelium-derived contracting factor (Joshua *et al*., 2005). It has been also reported that endothelium-dependent hyperpolarization was reduced by diabetes in rat mesenteric arteries. This factor hyperpolarizes the underlying smooth muscle via activation of K+ channels that relaxes smooth muscles (Quilley *et al*., 1997). In rat isolated perfused mesenteric vascular bed, it has been demonstrated that acetylcholine induced endothelium dependent vasodilation mediated by NO and EDHF (Yousif *et al*., 2002). Other studies indicated that exercise training could induce angiogenesis and improve vasculature responsiveness in different vascular beds (Delp, 1995; Laughlin, 1995) increasing eNOS protein (Sessa *et al*., 1994; Johnson *et al*., 2001) and NO production (Laughlin, 1995). In spite of these beneficial effects of exercise training, it has been reported that exercise especially severe exercise could act as pro-oxidant (Fisher-Wellman and Bloomer, 2009) that may challenge with its effectiveness in reduction of endothelial dysfunction. It has also reported that exercise induces oxidative stress and at the same time potentiates antioxidant defense system (Radak *et al*., 2008).

GSE is known as a powerful antioxidant and could improve diabetes-induced endothelial dysfunction (Shi *et al*., 2003). It has also an endothelial dependent vasodilation effect by phosphorylation of eNOS and increased NO production (Edirisinghe *et al*., 2008). In addition, it has been shown that GSE procyanidins could inhibit calcium release and blocks voltage-dependent calcium channels that could results in vasorelaxation and decreases arterial blood pressure (Zhang *et al*., 2008). Treatment of diabetic rats with antioxidants such as α-lipoic acid (Cameron *et al*., 2001) Quercetin (Machha *et al*., 2007) and 3', 4'-Dihydroxyflavonol (Woodman and Malakul, 2009) had marked beneficial effects on both NO and EDHF mediated endothelium-dependent relaxation of mesenteric vasculature.

It seems that, this is the first report showing the beneficial effects of combined ET and GSE administration on vascular function in diabetic rats. The more increase in plasma anti oxidant capacity and improvement of endothelial function of TrExD group shows that this combination is more effective than administration of ET or GSE alone and several mechanisms may be involved. As mentioned above, both interventions could improve endothelial function through different pathways and mechanisms to alleviate STZ-induced endothelial

dysfunction. Combination of these two factors could potentiate these favorable effects, but the exact mechanisms and the involved systems need to be determined.

Because there was no significant difference in response to sodium nitroprusside in all groups, the impaired vasodilator response to acetylcholine in mesenteric vascular bed obtained from STZ induced diabetic rats may be results from endothelial layer and upstream of vascular smooth muscle. In supporting of this study several studies have shown that the vascular smooth muscle response to sodium nitroprusside was not changed (Cameron *et al*., 2001; Chakraphan *et al*., 2005; Fatehi-Hassanabad *et al*., 2006; Inkster *et al*., 2007). In contrast, another study have shown that the endothelium independent relaxation of mesenteric vascular beds in 6 months STZ induced diabetic rats has been changed and may be attributed to the duration of diabetes in this study (Olbrich *et al*., 1996).

Vascular responses to phenylephrine have not changed by STZ induced diabetes or ET and/or GSE treated animals. There are some reports indicated that the vasoconstrictive response to phenylephrine of mesenteric vascular bed of Sprague Dawely diabetic rats (Inkster *et al*., 2007) or renal artery rings response of type 2 diabetic mice (Rodriguez *et al*., 2006) were reduced. In contrast, there are some other studies shown that responses of aortic rings obtained from 4 weeks diabetic rats (Majithiya and Balaraman, 2006) type 2 diabetic rats (Ozer *et al*., 2006) and 8 weeks alloxan induced diabetic rats (Akar *et al*., 2011) to phenylephrine increased. These discrepancies in the results may be attributed to the duration of diabetes, type of diabetes and vascular bed studied.

CONCLUSIONS

In conclusion, the data indicated that ET combined with GSE administration had more significant improving effect on plasma antioxidant capacity, diabetic vascular endothelial function and blood glucose than ET or GSE alone and may constitute convenient and inexpensive therapeutic approach to diabetic vascular complications.

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