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Effects of flaxseed intake on vascular contractile function in diabetic rats

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In diabetes, one of the most important causes of morbidity and mortality is vasculopathy. Though flaxseed (FXS) is known for improving cardiovascular health, only limited studies are available on FXS concerning diabetic vascular reactivity. Hence, in this study, we studied vascular reactivity changes after FXS treatment on streptozotocin (STZ)-induced diabetic rat aortae. Female Wistar rats were divided into following four groups: control (C), FXS treated (CT), diabetic (D), and FXS treated diabetic (DT) groups. FXS (0,714 g/kg/day; orally) was started after one week of STZ injection and was given for 12 weeks, phenylephrine (Phe)-induced contractions were obtained on isolated aortic rings in the presence of indomethacin, L-NAME and superoxide dismutase (SOD), individually. Phe-responses were increased significantly in D group and completely improved after FXS intake, whereas FXS increased vascular reactivity to Phe in C group. Indomethacin incubation significantly attenuated Phe-induced contractions in all groups of aorta, particularly in D group. L-NAME incubation significantly increased Phe responses in all groups except D group. SOD incubation decreased the contractions efficiently in D group. The decreament was much lower in DT compared to D group, but reverse effects were observed in CT group. Our findings suggest FXS may provide beneficial effects on diabetes-induced vascular reactivity changes through NO and prostaglandin dependent pathways, but in healty condition FXS may have adverse effect probably via pro-oxidant activity.

Keywords: Diabetes, Linseed, *Linum usitatissimum*, Vasculopathy

Diabetes mellitus (DM) is associated with an increased risk of cardiovascular disease such as coronary heart disease, peripheral arterial disease and hypertension. Oxidative stress (OS) is a feature of DM and vascular endothelium is a major target of OS playing a critical role in the pathophysiology of vascular diseases, and its alteration significantly contributes to diabetic vascular pathology. Endothelial dysfunction is characterized by a reduction of vasodilators and/or an increase in endothelium-derived contracting factors (EDCFs) in DM. Although there are different mechanisms that induce the process of endothelial dysfunction in DM, hyperglycemia is the main mediator. Observations suggest that the damage from hyperglycemia on endothelium is secondary to OS[1-4].

Flaxseed (FXS), *Linum usitatissimum* (Linn.) (Linaceae) (flax), also known as linseed, have various active compounds which make FXS an important plant in traditional medicine. It has various beneficial effects in dyslipidemia, cardiovascular diseases and diabetes[5-9]. From a nutritional perspective, flax is a well-known plant because of its oil, protein, fiber, lignan content and others. FXS is rich in α-linolenic acid (ALA) and secoisolariciresinol diglucoside (SDG) that have cardioprotective and anti-atherogenic properties[5,10-12]. The available data sustain that the cardioprotective properties of FXS, are due not only to hypocholesterolemic effects but also to several ways like antioxidative, anti-inflammatory and antithrombotic effects, and previous studies reported that FXS intake improve glycemic control in diabetes[6,7,13-15]. We have already shown that 12-week FXS supplementation has beneficial effects in glucotoxicity via pentose phosphate pathway and glutathione-dependent enzyme activities in several tissues of streptozotocin (STZ)-diabetic rats[16]. Here, we studied the vascular reactivity results in aortae obtained from the same animals.

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Material and Methods

Animals, diabetes induction and treatment

Female Wistar rats (n=32) weighing 180-210 g were used. They were housed under standard laboratory conditions (21±2ºC, 12-h light/dark cycle) during experimental period. Standard pellet diet and water were supplied *ad libitum*. The experiments were approved by the Animal Care Ethics Committee of Ankara University. The principles of laboratory animal care (NIH publication No.85-23, revised 1985) were observed.

After 12 h starvation, diabetes was induced by i.p. injection of 40 mg/kg of STZ, freshly dissolved in cold citrate buffer, pH 4.5. Only citrate buffer was given to control rats. After one week following STZ application, blood was obtained from tail vein and glucose levels were measured using a hand-held glucometer (Roche Diagnostisc, Mannheim, Germany). Rats with glucose levels higher than 250 mg/dL were considered as diabetic[16]. They were randomly divided into four groups: Gr. I, Control (C; n=7; animals were given orally only vehicle:carboxymethyl cellulose (CMC: 0.01%) in distilled water for 12 weeks); Gr. II, Control treated (CT; n=9; animals treated orally with FXS 0,714 g/kg/day in vehicle for 12 weeks); Gr. III, Diabetic (D; n=7; animals were given orally only vehicle for 12 weeks); and Gr. IV, Diabetic treated (DT; n=9; animals treated orally with FXS in vehicle for 12 weeks). A flow diagram can be seen in Fig. 1.

Commercial FXS provided locally (Damiana Natural Products, Istanbul, Turkey, contained 20% omega-3 fatty acid) and they were crushed with a mixer, suspended with CMC in distilled water and immediately administered by an oral gavage at a dosage of 0,714 g/kg/day. This dose was choosen according to the previous clinical studies (50 g/adult (70 kg)/day)[17]. The same amount of CMC solution were given to the control group for the same period.

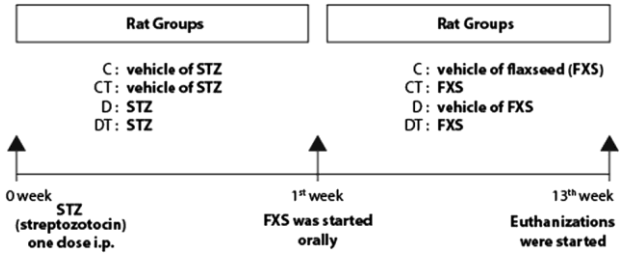

Fig. 1 — Schematic diagram of experimental groups and the treatments. [Groups: Control (C); control treated (CT); diabetic (D); and diabetic treated (DT)]

After 12-week treatment the rats were anesthetized, blood samples were collected from heart, the plasma were stored at –80° C. Thoracic aortae were attentively cleaned from connective tissue and cut into pieces approximately 4 mm in length for vascular reactivity studies.

Vascular reactivity studies

The aortic rings were hanged on a organ bath at 37°C containing 20 mL Krebs' solution (in mM: NaCl 118, KCl 4.7, NaH_2PO_4 1.2, $NaHCO_3$25, $MgSO_4$.7H20 1.2, glucose 11.2, $CaCl_2$ 2.5) aerated with 95 % O_2, 5 % CO_2 mixture (pH 7.4). The rings were streched to an optimal resting tension of 2 g and were equilibrated for 60 min[18]. The isometric tension was recorded using a force displacement transducer connected to an acquisition system (Commat Ltd., Ankara, Turkey). At the end of the equilibration time, aortic rings were exposed twice to 60 mM KCl to check the rings' functions. Subsequently, the endothelial integrity was examined using acetylcholine (Ach: 10 mM) on rings pre-contracted with phenylephrine (Phe; 10^{-6} M). After washout time (30 min) cumulative concentration-response curves with Phe (10^{-9} - 10^{-4} M) were obtained in all group of rings. After the experiment the tissues were allowed to recuperate for 30 min and the solution was replaced every 15 min before any protocol was started. To determine the vasoconstrictor effect of cyclooxygenase (COX) pathway, the rings were incubated with 10^{-5} M indomethacin (INDO: an inhibitor of cyclooxygenase derived prostanoids synthesis) 20 min before cumulative Phe applications. The other protocols were done with incubating L-nitro arginine methyl ester (L-NAME; a non selective nitric oxide synthase, 10^{-5} M) to examine the role of NO (nitric oxide) released from endothelial cells on Phe contractility, and superoxide dismutase (SOD; 1500U; inactivates superoxide radicals and increases bioavailability of NO) for 20 min before cumulative Phe applications. Endothelium-independent relaxations were also investigated using sodium nitroprusside (SNP: 10^{-11} - 10^{-4} M) in pre-contracted aortae

Chemicals

All chemicals were analytical grade and purchased from Sigma-Aldrich (St.Louis, USA) or Merck (Germany).

Statistical analysis

Statistical analysis was carried out by one-way ANOVA followed by *post hoc* Newman-Keuls test using GraphPad Prism 5.00 for Windows (GraphPad

Software, USA), *P <0.05, **P <0.01, ***P <0.001 were considered significant.

Results and Discussion

Body weight, blood glucose and triglyceride levels in the groups can be seen in our previous article[16].

Vascular reactivity results

FXS treatment normalized the Phe contractile profile on aortae from D rats (the contractions in DT group were similar to C group). But Phe contractile profile was increased significantly in CT group compared to C (and DT) groups. There was no statistical difference between CT and D groups (Fig. 2). After incubation with INDO, contractile responses to Phe significantly decreased in all aortic groups especially in D and DT groups (Fig. 3). After incubation with SOD, in CT group, at $3x10^{-8}$ - 10^{-5} M concentrations of Phe, significant differences were observed compared with CT before incubation ($p<0.001$). In incubated C group there were significant decreases in contractility, but not as much as CT group. After incubation with SOD, although both of D and DT groups showed significant decreases to Phe constrictor responses, less constrictor effect was observed in D group (Fig. 4). Incubation of aortic rings with L-NAME, significantly increased the Phe vasoconstrictor responses in C, CT and DT groups, but in D group no significant change was observed (Fig. 5). No significant changes were observed in endothelium-independent relaxations induced by SNP (10^{-11} - 10^{-4} M) among the groups (n=5) (data not shown).

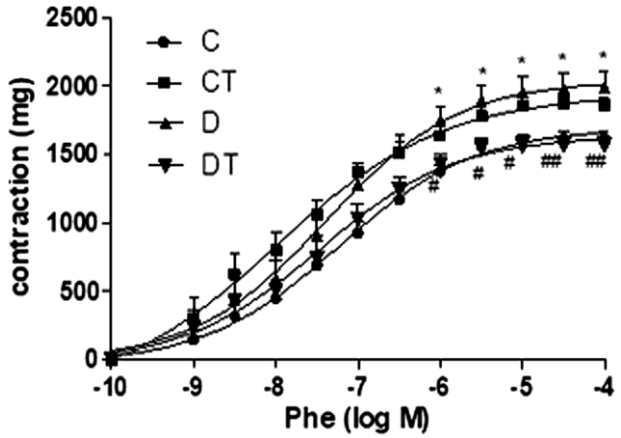

Fig. 2 — The cumulative concentration-response curve of phenylephrine (Phe) on aortic rings. [Values are mean ± SEM. Control (C) group (n=7), control treated (CT) group (n=9 each), diabetic (D) group (n=7), diabetic treated (DT) group (n=9). *P <0.05 *vs*. C group, $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs*. D group)]

In the present study, we have demonstrated that FXS has beneficial effects on impaired vascular reactivity in diabetic state, but not in healthy rats. It is known that diabetes triggers endothelial dysfunction, impaired vascular reactivity and atherosclerosis. In diabetic endothelial dysfunction, decrement in endothelium-dependent relaxing factors (EDRFs) and increment in EDCFs lead to vascular tone enhancement, platelet aggregation, and thrombus. OS increases in diabetic state in consequence of reactive oxygen species' (ROS) overproduction together with decrease in antioxidative systems leading endothelial dysfunction. ROS causes reduction on nitric oxide (NO) bioavailability, and increases the effect of EDCFs. As a consequence, the endothelial balance is inclined to vasoconstrictor responses in diabetes[4,19-21].

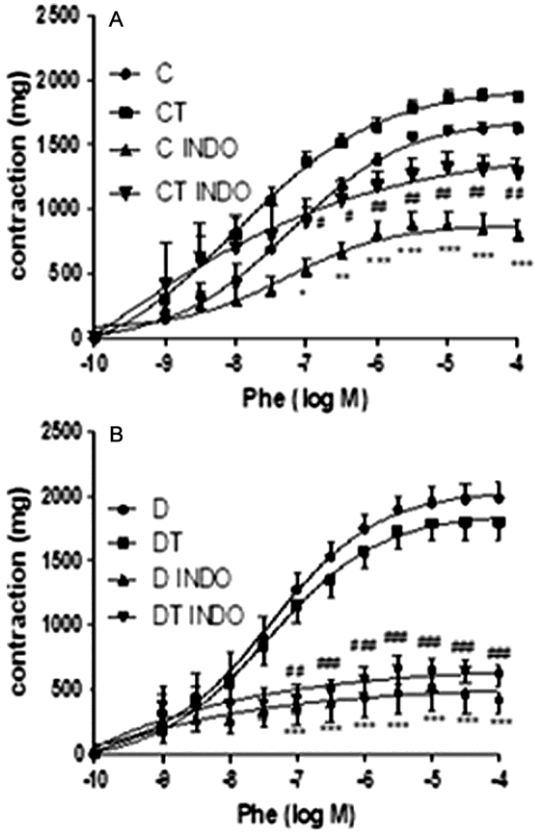

Fig. 3 — The cumulative concentration-response curves of phenylephrine (Phe) alone and in presence of indomethacin (INDO) on aortic rings. [Values are mean ± SEM. Control (C) group (n=7), control treated (CT) group (n=9). C INDO group's statistical differences were shown as *P <0.05, **P <0.01,***P <0.001 *vs*. C group; CT INDO group's statistical differences were shown as $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs*. CT group. Diabetic (D) group (n=7), diabetic treated (DT) group (n=9). Statistical differences of D INDO group *vs*. D group were shown as ***P<0.001 and DT INDO group *vs*. DT group were shown as $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001]

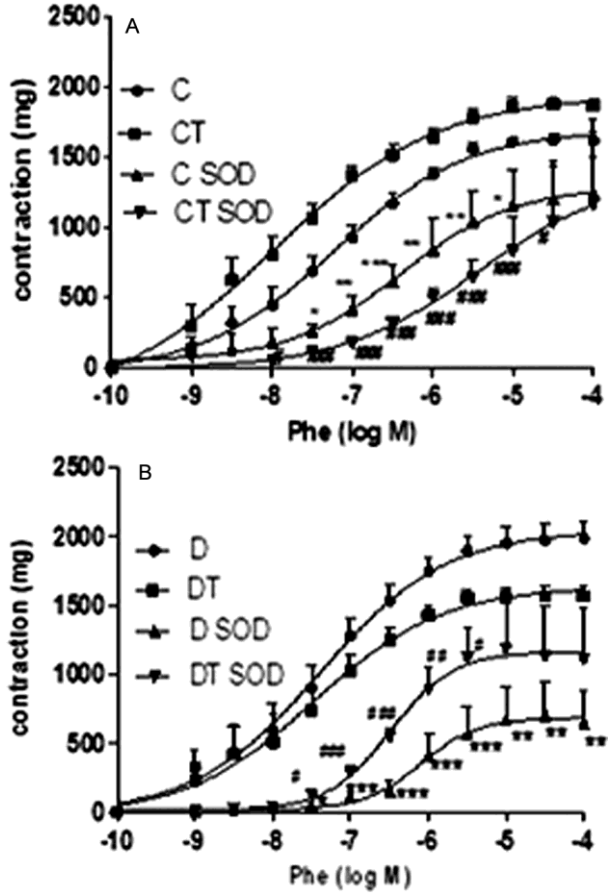

Fig. 4 — The cumulative concentration-response curve of phenylephrine (Phe) alone and in presence of superoxide dismutase (SOD) on aortic rings. [Values are mean ± SEM. Control (C) group (n=7), control treated (CT) group (n=9). Statistical differences of C SOD group were shown as **P* <0.05, ***P* <0.01, ****P* <0.001 *vs*. C group; CT SOD group were shown as $^{\#}P$<0.05, $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001 *vs*. CT group. Diabetic (D) group (n=7), diabetic treated (DT) group (n=9). Statistical differences of D SOD group vs. D group were shown as **P* <0.05, ***P* <0.01, ****P* <0.001; DT SOD group *vs*. DT group were shown as $^{\#}P$ <0.05, $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001]

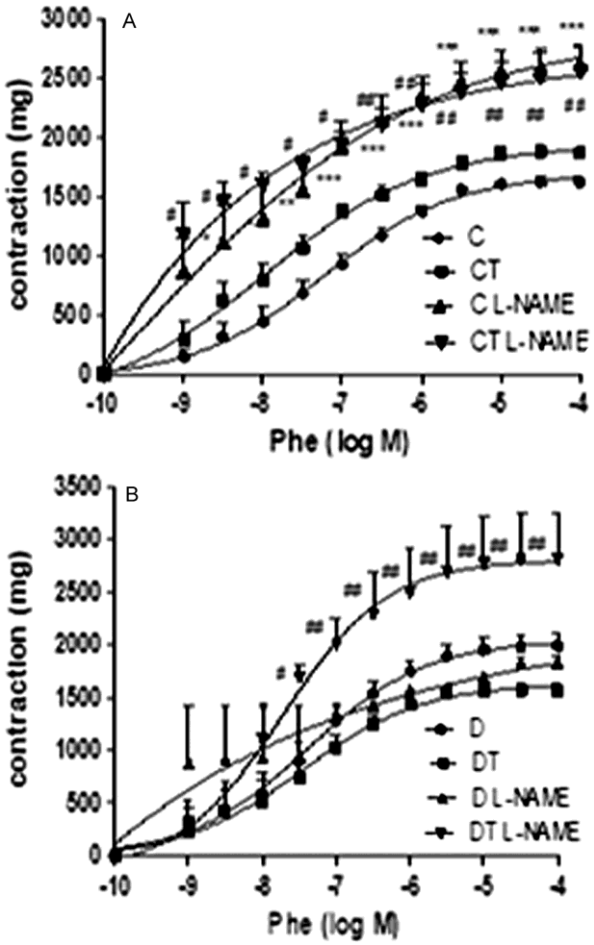

Fig. 5 — The cumulative concentration-response curve of phenylephrine (Phe) alone and in presence of L-NAME on aortic rings. [Values are mean ± SEM. Control (C) group (n=7), control treated (CT) group (n=9). Statistical differences of C L-NAME group were shown as **P* <0.05, ***P* <0.01, ****P* <0.001 *vs*. C group; CT L-NAME group were shown as $^{\#}P$ <0.05,$^{\#\#}P$ <0.01 *vs*. CT group. Diabetic (D) group (n=7), diabetic treated (DT) group (n=9). DT L-NAME group vs. DT group were shown as $^{\#\#}P$ <0.01]

Diabetic (D) rat aortae showed increased vasoconstrictor responses to Phe relative to controls (C), in agreement with previous studies[18,20]. FXS treatment improved this defective responsiveness. It is known that EDCFs involve activation of cyclooxygenases (COXs) and release certain prostanoids that stimulate thromboxane (TX) prostanoid receptors (TPRs). The stimulation of TPRs elicits vascular contraction, vascular smooth muscle cell proliferation, platelet aggregation and inflammation. Additionally, the generation of endothelial vasodilator-antiaggregant prostacyclin (PGI_2) via COX-1 decreases, and generation of vasoconstrictor-aggregant TXA_2 in endothelium and in platelets increases in diabetic state. This imbalance of the PGI_2/ TXA_2 ratio partially account for atherogenesis in diabetes, and COX-1 and COX-2 produce vasoconstrictor prostanoids in diabetic aorta[3,22,23]. Thus, first, the effect of prostanoids' role on increment in Phe responses with FXS treatment in our diabetic aortae is investigated. After incubation with non-selective COX inhibitor indomethacin (INDO), all of the aortic groups showed significant decrease to Phe constrictor responses, especially D group. Although significant decrease occured in DT group, maximum decrease was observed in D group. Further, when compared to CT group, a significant decrease was observed in CT INDO group and also when compared to C group, Phe responses decreased

in C INDO group significantly. These results suggest that much more vasoconstrictor COX products are generated in D group aorta and FXS intake reduced these COX products in D group.

It has been reported that α-linolenic acid (ALA) decreases TXA_2 and increases PGI_2 in vessels[3,24]. This may enhance endothelium-dependent vasodilation. Additionally, the inhibitory effect of FXS oil on platelet aggregation is indicated in rabbits[25]. Thus, TX plays a role on vascular dysfunction in diabetic state. Notably, in the present study, inhibition of the COX pathway with INDO decreased aortic contraction to Phe in D group much more than C group. ROS production has a major role in the development of vascular dysfunction in diabetes increasing the vasoconstrictor eicosanoids' generation and tends to show more inflammatory, vasoconstrictor, pro-thrombotic effect[3,19]. A decrement was reported in EDRFs responses associated with enhancement in COX-1, COX-2 expression, TXA_2, PGE_2 and OS in type 2 diabetic rat mesenteric arteries[26]. It was concluded that endothelial dysfunction in diabetes can be reversed in part by treatment with eicosapentaenoic acid (omega-3 fatty acid), TXA_2 synthase inhibitor and antioxidant[3]. Upregulation of protein kinase C (PKC) mediated COX-2 is related with the increment of TXA_2 and decrement of PGI_2 release in diabetes[4]. Hyperglycemia increases PKC activity and this upregulates peroxidase enzymes and COX pathway, with resultant excessive generation of ROS/nitrogen species. This process was amplified when the antioxidative systems become compromising. All these cellular mechanisms provide support for the usage of antioxidants to prevent diabetic complications[1,2].

FXS lignan has antioxidant properties as a scavenger of several free radicals, such as superoxide (O_2^-), hydroxyl ($OH^\bullet$) and hydrogen peroxide (H_2O_2)[27-29]. In the present study, Phe contractions were reduced more in the presence of SOD in D group aortae than that of DT group. Also, more reduction was observed in aortae from CT group than C group aortae after SOD incubation. On the other hand, Nunes *et al*.[30] stated that 15 days of FXS oil treatment in healthy rats adversely increases aortic vascular reactivity to Phe by increasing COX-2 mediated TXA_2 and ROS generation. It is mentioned that ALA can easily be oxidized, and FXS oil can also cause peroxidation of lipids which may adversely affect the vessels, and linoleic acid (LA) can increase PG_2s and TX, which augments vascular tonus. These results could explain why Phe contractions were increased in CT group in our study and SOD incubation was more effective in aortae from CT group than that of C group. It is known that an agent can be both pro- or anti-oxidant according to physiological state. FXS contains fatty acids, such as polyunsaturated fatty acids including ALA and LA, dietary fibers and phytoestrogen lignans, and because FXS posesses antioxidant potential it can be thought that FXS exerts important antioxidant effects, but, this is also has not been the case in some clinical studies. For example, two clinical trials have reported that 3 weeks of FXS (50 g/day) or 4 weeks of FXS oil (20 g/day) treatment caused an increase on OS markers in hyperlipidemic adults, and LDL oxidation was observed in obese patients[31,32]. The effects of FXS on contractile and vasodilator responses are also contradictory in animal model experiments. For eg., for 6, 8 or 16 weeks of 10% FXS supplementation does not alter norepinephrine-induced vasoconstriction or Ach-induced relaxation responses in hyper-cholesterolemic rabbits[33]. However, another study reported that 20% FXS diet increases endothelial vasorelaxation in hypertensive rats[34].

EDRFs consist of nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF). Impaired endothelium derived vasodilation and basal NO-dependent vasodilatory tone is represented in several diabetic animals and in diabetic patients[2,4]. Several mechanisms are exposed for impaired endothelial NO responses in diabetic vessels, consisting of damages in signal transduction or substrate availibility, damage in the release of NO, increment in NO breakdown and increment in EDCFs. To investigate the effect of FXS intake on NO modulation of Phe-contractile responses, the aortic rings were incubated with L-NAME, a nitric oxide synthase (NOS) inhibitor. In D group, no significant change was observed by incubation, however Phe contractions were enhanced in DT-incubated group. These results may indicate that FXS treatment has no effect on NO production, but may improve vascular reactivity changes in DT group by reducing superoxide anion (O_2^-) generation and thus increasing NO bioavailability. Rapid destruction of NO occurs in diabetic vessels because of extensive OS, and it is well known that the increment in (O_2^-) is related with the accelerated break down of NO[2,4]. Thus, FXS may protect the vasculature by inhibiting the disruption of NO with antioxidant property in diabetes.

As a common observation, we found that sodium nitroprusside (SNP)-induced relaxations in diabetic aortae (an endothelium-independent, cGMPmediated relaxant agent) were comparable to control aortae indicating FXS treatment did not affect vascular smooth muscle responsiveness.

FXS consists of plant lignans, and seco-isolariciresinol diglucoside (SDG) lignan in FX, and its metabolites possess potent antioxidant effect[35-37]. SDG has a scavenger activity on ROS. Moreover, it is reported that SDG has a role in delaying the development of diabetes in rat model of insulin resistance counteracting OS[37]. Further, synthetic SDG exert**s** anti-hyperglycemic effect in STZ- diabetic rats by protecting the liver from OS, and improves insulin sensitivity[38]. Hence, the improved endothelial vasodilation, as previously reported could be the effects of lignans in our diabetic animals. In type 2 diabetic rat model, dietary FXS oil (does not contain lignan and fibre) and fish oil reduces protein glycation and inflammation in liver[39,40]. Further, a daily FXS supplementation for 12 weeks in pre-diabetic obese or overweight individuals decreases glucose and insulin levels and improves insulin sensitivity[6]. We have already reported that FXS intake reduces glucose and TG levels in our diabetic rats, and has beneficial effects in glucotoxicity via regulating pentose phosphate pathway and glutathione-dependent enzyme activities in several tissues[16]. Anti-hyperglycemic effect of FXS extracts has also been shown in alloxan or STZ diabetic rats and mice or hamsters fed with a high-fat diet[41-44]. However, as mentioned above, there are adverse clinical results on FXS oil supplementation which increases OS markers in hyperlipidemic or insulin resistant subjects. Also, a study on partially defatted FXS supplementation of hyperlipidemic subjects has reported significantly reduced serum thiol groups of proteins, possibly indicating oxidative activity[45]. Thus, the effects of FXS on markers of OS need further study. An agent can be both pro- or anti-oxidant according to dosage or physiological /pathological state and / or organ species. For example, vitamin C which is an antioxidant that also be a pro-oxidant[46].

Conclusion

The results of this study indicate that FX supplementation to diabetic rats improves diabetes-induced vascular reactivity changes to vaso-constriction and endothelial dependent relaxation by increasing NO bioavailability and modulating PG dependent pathways. Thus, FXS may protect the vasculature with antioxidant property in diabetes but, in healthy state FXS supplementation may augment vascular reactivity to vasoconstrictors. To the best of our knowledge, this is the first study to identify the favourable effect of the FXS supplementation on vascular reactivity in STZ-diabetic rats. More research is needed to clarify the mechanism (s) for beneficial effects of FXS in diabetic vascular reactivity, and also possible vascular adverse effects in healthy state. Although the clinical significance of our results is not clear, healthy people should avoid uncontrolled use of FXS supplements.

Conflict of interest

Authors declare no competing interests.

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