

Full Length Research Paper

Influence of defatted flaxseed diet on insulin sensitivity, vascular permeability and lipid profile in a rat model of type 2 diabetes mellitus

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The impact of flaxseed (FSD) intake, a rich source of alpha linolenic acid (ALA), fiber and lignans, on the cardiovascular system is well documented. However, mechanisms by which flaxseed improve cardiovascular health are not clear. The aim of this study was to investigate the effects of dietary flaxseed on vascular permeability and endothelial function in streptozotocin-induced type 2 diabetes in rats. Type 2 diabetes mellitus (T2DM) was induced in male Sprague-Dawley rats by intraperitoneal injection of streptozotocin (35 mg/kg body weight) after short term feeding of high fructose diet. Diabetic rats were divided into three groups, one group fed standard diet, second group fed standard diet supplemented with defatted flaxseed powder (FSD), and third group received Metformin (200 mg/kg BW (body weight)) for 8 weeks. Fasting serum concentrations of glucose (FPG), insulin, vascular endothelial growth factor (VEGF), nitric oxide (NO), uric acid (UA) and lipid profile were measured. Vascular permeability index (VPI) was assessed at the end of experiment by quantifying the extravasation of albumin-bound Evans blue (EB) dye in the heart. Dietary FSD supplementation is comparable to Metformin in modulating blood lipid profiles, insulin and FPG levels. FSD intake was associated with significant reductions in serum insulin (-66%), glucose (-68%), VEGF levels (-66.8%) and UA (-63%), and NO (-37.5%) as compared to diabetic group without FSD supplementation. There were also improvements in lipid profile, vascular permeability index (VPI), insulin resistance and atherogenic indices in diabetic rats supplemented with flaxseed. These results suggest that dietary FSD supplementation may reduce the incidence of diabetic vascular complications through improvement of insulin sensitivity, vascular permeability and lipid profile.

Key words: Diabetes mellitus, cardiovascular diseases, vascular permeability, endothelial dysfunction, insulin resistance, vascular endothelial growth factor, nitric oxide.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a progressive and complex metabolic disease characterized by hyperglycemia and a combination of insulin resistance (IR) and an inadequate compensatory insulin-secretory response (Tsuchiya et al., 2007). More recently, IR has been shown to be an independent risk factor for the development of coronary atherosclerosis and acute cardiovascular events (Mita et al., 2010). Endothelial

dysfunction has been found to be associated with IR (Kahn et al., 2011; Tsuchiya et al., 2007). Dysfunction of the vascular endothelium is regarded as an important factor in the pathogenesis of diabetic micro- and macroangiopathy. Moreover, it has become apparent that endothelial dysfunction is not restricted to patients with T2DM but is also present in individuals with IR or who are at high risk for developing T2DM (Caballero et al., 2008; Valle Jimenez et al., 2007).

It has also been suggested that IR may exert a deleterious effect on endothelial function as a result of reduced insulin-mediated vasodilatation and endothelium-derived nitric oxide production (Han et al.,

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2011; Xiang et al., 2008). Endothelium-derived NO is thought to antagonize the stimulatory effect of vascular endothelial growth factor (VEGF) on expression of adhesion molecules such as E-selectin, and vascular cellular adhesion molecule, thereby protecting the endothelium from the atherogenic effects of excessive interactions with circulating monocytes (Zhang et al., 2009). In IR state, insulin is no longer capable of stimulating NO production or antagonizing VEGF, leading to endothelial dysfunction, disrupted vascular permeability and atherogenic effects (Wang et al., 2004). Targeting VEGF may prove useful as a therapeutic strategy for the treatment of early diabetes, however since VEGF is vital in processes such as angiogenesis in the myocardium and wound healing (Ferrara, 2001; Holmes and Zachary, 2005), systemic therapies against VEGF are not feasible.

Previous studies reported flaxseed is a rich source of alpha linolenic acid (ALA), the plant-based omega-3 (n-3) fatty acid, fiber and lignans, making it a potentially attractive functional food for modulating cardiovascular risk (Rodriguez-Leyva et al., 2010). Moreover, in-vitro and in-vivo experimental anticancer studies indicate that flaxseed decrease extracellular levels of VEGF without agonistic effects (Bergman Jungstrom et al., 2007; Dabrosin et al., 2002), and decrease tumor angiogenesis in estrogen-dependent breast cancer (Chen et al., 2011; Lindahl et al., 2011). However, the impacts of flaxseed on NO, VEGF and vascular permeability during IR and T2DM states are unknown. Therefore, the present study aims to evaluate the impact of consumptions of ground flaxseed on ameliorating IR-induced vascular permeability dysfunction in a model of T2DM in rats.

MATERIALS AND METHODS

Experimental drugs and diet

Streptozotocin was purchased from Sigma-Aldrich (Sigma chemicals Co., St. Louis, MO, U.S.A.). It was dissolved in ice-cold 0.05 M citrate buffer (pH 4) immediately before use. Metformin HCl (Glucophage 500 MG tab) was obtained from Merck Sante Corporation (France). Metformin was dissolved in distilled water containing 0.9% (wt/vol) sodium chloride for oral administration.

The flaxseed diet was prepared by the addition of 15% ground defatted flaxseed powder to standard rat chow as previously described (Bergman Jungstrom et al., 2007).

Experimental design

Male Sprague-Dawley rats (160 to 220 g body weight, 12 weeks old) were used in this study. Rats were kept for 2 weeks on balanced ration with water *ad libitum* for acclimatization. All experiments were clearly justified and approved by the Committee on Animal Care and Use of the university. Induction of T2DM was done as previously described (Elcioglu et al., 2010). Briefly, after 2 weeks feeding with 10% w/v fructose solution *ad libitum* in feeding bottle, low dose of 35 mg/kg streptozotocin (Sigma Chemical Co, MO, USA) dissolved in 1 ml of 0.05 M citrate buffer was administered intraperitoneally. Then rats were fed with conventional

laboratory chow.

Ten healthy control rats and 30 impaired glucose tolerance (IGT) rats as determined by oral glucose tolerance test (OGTT) were divided into four groups (n = 10 each) as the following:

- Group 1: Healthy control rats (NC) included rats that were fed with standard rat chow and tap water *ad-libitum*;
- Group 2: Diabetic untreated rats (T2DM) that were kept without treatment;
- Group 3: T2DM-Metformin treated rats (MET) which were treated daily with 200 mg/kg BW of Metformin hydrochloride using oral gavage;
- Group 4: T2DM-flaxseed diet fed rats (FSD).

Rats were weighed weekly throughout the study. At the end of Week 6, blood samples from a retro-orbital sinus puncture were taken and blood serum were separated and stored at -20°C until further analysis.

Biochemical measurements

Blood serum samples were subjected for analysis of total plasma cholesterol, high-density lipoprotein cholesterol (HDL-C), low density lipoprotein (LDL), triglycerides, UA and glucose were assayed with an automated analyzer, Dade Behring Dimension RxL clinical chemistry system (Dade Behring; Germany) using its own kits. Serum insulin concentrations were determined using rat insulin Enzyme Linked Immunosorbent Assay (ELISA) kit (ALPCO, NH, USA). Serum nitrite and nitrate content as a measure of NO was measured using NO assay kit (Calbiochem, Darmstadt, Germany). VEGF was determined in serum samples using rat VEGF immunoassay (Quantikine rat VEGF EIA, R&D systems, MN, USA) according to manufacturer instructions.

Atherogenic index of plasma (AIP)

The atherogenic ratio of TC/HDL-C as well as the AIP, calculated as $\log(\text{TG}/\text{HDL-C})$, with TG and HDL-C, were measured in molar concentrations (Dobiasova and Frohlich, 2001).

Homeostasis model assessment of insulin resistance index (HOMA-IR)

The HOMA-IR was calculated from plasma fasting insulin and glucose concentration according to the Matthews formula (Matthews et al., 1985):

$$\text{HOMA-IR} = \text{Plasma fasting insulin (uU/L)} \times \text{Plasma fasting glucose (mmol/L)} / 22.5$$

Vascular permeability index (VPI)

Microvascular permeability to albumin was assessed at the end of experiment by quantifying the extravasation of albumin-bound EB dye in the heart muscle. Unanesthetized animals were injected with Evans blue dye (20 mg/kg) in the caudal vein 10 min before being killed and EB dye was extracted by formamide from heart tissue collected after exsanguinations. Half of each tissue sample was dried at 60°C for 24 h, and a dry/wet weight ratio was calculated to avoid underestimation of EB dye concentration due to local edema. The other half was placed in a formamide solution (4 mL/g wet tissue) for 24 h for dye extraction. The extracted amount of EB dye was determined by spectrophotometry at 620 nm using a 96-well microplate photometer (Chakir et al., 1998). VPI was calculated as

Table 1. Effect of different treatments on body weight gain, glucose, insulin and HOMA-IR.

Parameter\group	Body Wt gain (g)	FPG (mmol/L)	FPI uU/L	HOMA-IR
Group 1 (NC)	42.40±13.75	4.99±0.13	5.96±2.72	1.16±0.33
Group 2 (T2DM)	86.00±17.45	19.50±0.76	18.76±5.44	6.04±0.25
Group 3 (MET)	25.00±3.39 ^{###}	5.70±0.98 ^{**}	9.12±3.24 [#]	2.75±0.36 ^{###}
Group 4 (FSD)	47.60±18.72 [*]	6.27±0.63 [*]	6.44±2.48 ^{**}	1.78±0.48 ^{**}

Values are expressed as means ± SD (n=10). #, p<0.05, ##, p<0.01 compared with healthy group; *, p<0.05, **, p<0.001 compared with untreated diabetic group. Group NC: Normal healthy controls; group T2DM, untreated diabetics; group MET, metformin-treated diabetics; group FSD, flaxseed-treated diabetics.

Table 2. Effect of different treatments on some markers of endothelial functions.

Parameter\group	VPI (ug/g)	NO (umol/L)	VEGF (pg/ml)	UA (mg/dl)
Group 1 (NC)	74.05±14.73	56.94±10.05	18.82±4.11	1.10±0.10
Group 2 (T2DM)	122.88±25.67	72.33±8.73	71.59±9.03	2.16±0.21
Group 3 (MET)	54.50±7.75 ^{##}	37.08±21.15 ^{##}	11.82±7.05 ^{##}	1.28±0.41 ^{**}
Group 4 (FSD)	81.50±11.86 [*]	45.24±9.72 [*]	21.37±6.21 ^{**}	0.89±0.11 ^{**}

Values are expressed as means ± SD (n=10). #, p<0.05, ##, p<0.01 compared with healthy group, *, p<0.05, **, p<0.001 compared with untreated diabetic group. NO, nitric oxide; VEGF, vascular endothelial growth factor; UA, uric acid.

the ratio of EB dye concentration per dry weight of heart tissue, and it was expressed in micrograms/gram.

Statistical analysis

Data are expressed in terms of mean ± standard deviation (SD). To determine treatment effects and compare differences among group means, data were analyzed by One-way ANOVA (analysis of variance) followed by post-hoc Duncan's multiple range test. Statistical significance was accepted at P < 0.05. Data were analyzed using SPSS software version 13.0 for windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Two days after STZ injection, the blood glucose levels were measured and the animals with blood glucose levels higher than 12.5 mmol/L were considered to be diabetic and were included in the study. Body weight changes and biochemical analyses of blood samples withdrawn at the end of the fourth week are presented in Table 1. Rats of the untreated T2DM group showed significantly higher weight-gain, fasting blood glucose, insulin and HOMA-IR compared with NC group. Group treated with MET showed significant decrease (p<0.01) in weight gain, FPG levels (p<0.05), FPI (p<0.01) and HOMA-IR. Treatment with FSD has been found to show comparable efficacy in lowering FPG and FPI, however, FSD fed group showed lower HOMA-IR ratios than Metformin-treated group.

Effect of different treatments on NO, VEGF and UA as markers of endothelial functions are represented in Table

2. Untreated diabetic group showed significantly higher concentrations of serum VEGF, NO and UA as compared to NC group. Serum concentrations of VEGF (both p<0.01) and UA (p<0.05, 0.01 respectively) and NO concentrations were significantly decreased (p<0.001, 0.05 respectively) after either treatments compared with untreated T2DM group. Treatment with MET or FSD significantly lowered VPI (p<0.01, 0.05, respectively) (Figure 1).

Table 3 represents the impact of different treatments on lipid profile and atherogenic indices. There were significant elevations in levels of serum cholesterol, triglycerides, LDL and significant decrease in HDL levels in T2DM group. After four weeks of treatment, triglycerides, LDL and HDL levels were restored to normal values of control group. Metformin-treated group showed significantly lower values of serum cholesterol compared to FSD group. Figure 2 represent the improvement of the atherogenic index post treatment with Metformin and FSD.

DISCUSSION

Impact of FSD on hyperglycemia and IR

The present investigation showed an increase in plasma glucose level associated with hyperinsulinaemia in untreated rats that confirms the induction of insulin resistance/type 2 diabetes mellitus. The degree of IR was also significantly increased as indicated by high HOMA-IR values. Treatment with FSD has been found to show comparable efficacy in lowering FPG and FPI, however,

Table 3. Effect of different treatments on parameters of lipid profile.

Parameter/group	Cholesterol (mmol/L)	Triglycerides (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)
Group 1 (NC)	2.95 ± 1.05	1.39 ± 0.27	1.53 ± 0.65	1.12 ± 0.21
Group 2 (T2DM)	5.67 ± 1.46	2.77 ± 0.39	3.51 ± 1.23	0.41 ± 0.17
Group 3 (MET)	2.33 ± 0.91**	1.24 ± 0.29**	1.13 ± 0.46**	1.09 ± 0.14**
Group 4 (FSD)	2.76 ± 1.04**	1.33 ± 0.16**	1.17 ± 0.68**	0.99 ± 0.16**

Values are expressed as means ± SD ($n=10$). #, $p<0.05$, ##, $p<0.01$ compared with healthy group, *, $p<0.05$, **, $p<0.001$ compared with untreated diabetic group. LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol.

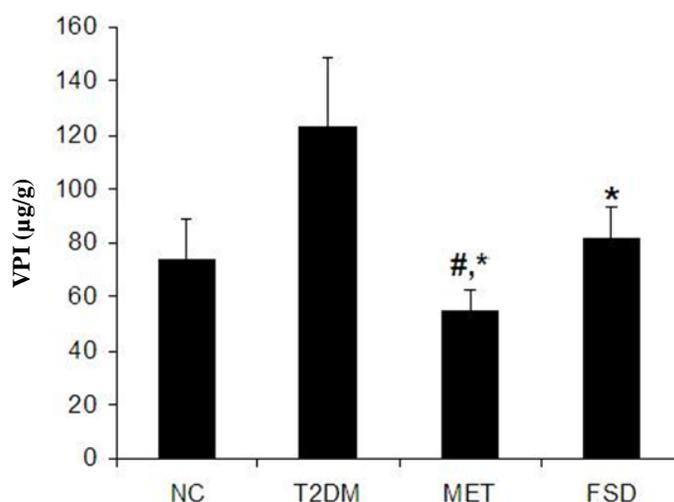


Figure 1. Improvement of vascular permeability index (VPI) post treatment with FSD. VPI, Vascular permeability index, is the ratio of EB (in µg) per dry weight of heart tissues (in g). #, $p<0.05$ compared with healthy group, *, $p<0.01$ compared with untreated diabetic group.

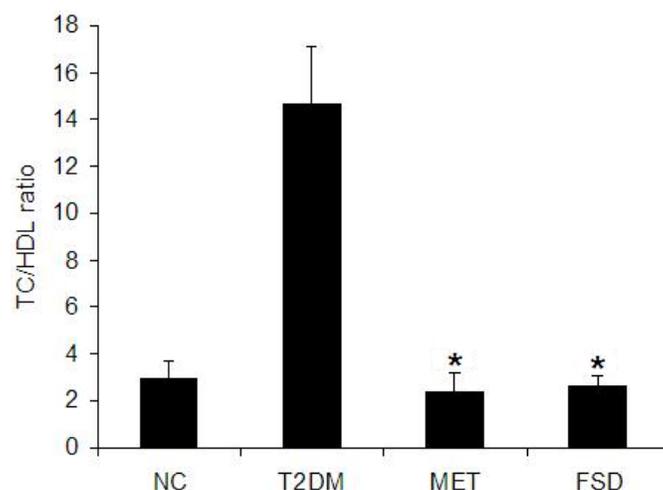


Figure 2. Improvement of atherogenic index (TC/HDL ratio) post treatment with FSD. VPI, Vascular permeability index, is the ratio of EB (in µg) per dry weight of heart tissues (in g). *, $p<0.01$ compared with untreated diabetic group. HDL-C: high density lipoprotein cholesterol; TC, total cholesterol.

FSD fed group showed lower HOMA-IR ratios than Metformin-treated group.

Impact of FSD on endothelial permeability, NO and VEGF

The increase in IR and hyperglycemia were coupled with significant increase in vascular hyperpermeability as indicated by increase in serum VEGF concentration, microvascular protein extravasation as evidenced by increased VPI, and an increase in serum NO concentrations.

Recent evidence in experimental animals and humans shows that nitric oxide (NO) plays a key role in insulin transendothelial permeability of glucose and cardiovascular homeostasis (Chien et al., 2005). However, it is still controversial that patients with type 2 diabetes have a decreased ability of insulin to increase endothelial nitric oxide (NO) release. Recent evidence indicates that higher levels of NO are present in subjects with diabetes (Ghasemi et al., 2011). This increase in NO levels could be an effort to compensate for the defect in subsequent signal transduction. In this way, even though the NO concentration is increased, its biological effect is still impaired. Studies in diabetic patients have demonstrated impaired endothelium-derived NO activity with or without normal response to exogenous NO (Cleland et al., 2000) and in diabetic animals (Chien et al., 2005). In accordance with these studies, we found NO levels are significant higher in T2DM than control animals.

The animal group supplemented with FSD showed normal concentrations of NO comparable to NC group and to that of Metformin treated group, suggesting that it improved NO bioavailability. This observation was also associated with normalization in vascular permeability as indicated by lower protein extravasation in the heart tissue than in untreated diabetic group. These results indicate that supplementation with FSD in animal model of T2DM could improve endothelial dysfunction and ameliorate IR-induced vascular hyperpermeability observed in untreated T2DM group. These findings are in concert with the demonstrations that flaxseed diet improves endothelial vasorelaxant functions (Bergman Jungstrom et al., 2007).

In addition, our findings confirm previous report that documented the ability of flaxseed in inhibiting extracellular levels of the angiogenic factor VEGF (Bergman Jungstrom et al., 2007; Dabrosin et al., 2002). VEGF appears to be in the pathway whereby inflammation leads to cardiomyopathy and death (Mallamaci et al., 2008). Moreover, previous studies have shown that hyperglycemia induces up-regulation of VEGF and vascular permeability, ultimately leading to diabetes related CVD complications (Yamagishi et al., 2002). In the present study, these factors, VEGF, VPI and insulin, were recovered to normal values of control in flaxseed supplemented group, indicating that flaxseed consumption

may improve cardiovascular health through modulation of vascular hyperpermeability and hyperinsulinemia in T2DM.

Impact of FSD on lipid profile

In diabetes, dyslipidemia aggravates glucose effects both by inducing IR and by direct effects on beta-cells (Grill and Bjorklund 2009). Because both type 2 diabetes and elevated plasma lipid levels are important independent risk factors for CVD and coronary heart disease, the choice of an anti-hyperglycemic agent for patients with type 2 diabetes should take into account effects on lipids as well as on markers of glycemic control. It is evident from our results that FSD performed better in terms of lowering total atherogenic index, LDL-C, triglycerides and increasing HDL-C than Metformin. These results are in concordance with previous studies (Lucas et al., 2004; Mandasescu et al., 2005).

Improved insulin action in FSD-treated rats could be responsible for the normalization in lipid metabolism, and hence we observed normal plasma lipid profile in this group. However, previous studies show that flaxseed might also have a direct role in lipid metabolism (Cho et al., 2009; Haliga et al., 2007).

Hyperlipidemia has been implicated in the development of atherosclerosis (Drechsler et al., 2010; Van Craeyveld et al., 2011). There was also an improvement in the lipid atherogenic index in diabetic rats supplemented with flaxseed to a similar degree as Metformin. This result is in a good agreement with previously reported data which showed flaxseed intake reduces the progression of atherosclerosis to a greater extent, and tends to regress atherosclerosis (Bassett et al., 2009; Dupasquier et al., 2007; Prasad, 2008).

Conclusions

In conclusion, our findings indicate that flaxseed supplementation is comparable to Metformin in normalizing blood insulin, sugar and lipid profiles, and suggest that the mechanism by which flaxseed may improve cardiovascular health is based mainly on reducing VEGF, insulin resistance, augmenting NO bioavailability and normalizing lipid profile. Finally, considering the rapid global increase in the prevalence of type-2 diabetes and the high incidence of insulin resistance in the general population, inclusion of flaxseed in the daily diet could be recommended.

Abbreviations: AIP, Atherogenic index of plasma; ALA, alpha linolenic acid; ANOVA, analysis of variance; BW, body weight; CVD, cardiovascular; EB, Evans blue; ELISA, enzyme linked immunosorbent assay; FSD, flaxseed; FPG, fasting plasma glucose; FPI, fasting plasma insulin; HDL, high-density lipoprotein; IGT, impaired glucose tolerance; IR, insulin resistance; LDL, low density lipoprotein; NC, normal control; OGTT, oral

glucose tolerance test; **SD**, standard deviation; **UA**, uric acid; **VEGF**, vascular endothelial growth factor; **VPI**, vascular permeability index.

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