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Original Article

Effect of flaxseed on serum lipid profile and expression of NPC1L1, ABCG5 and ABCG8 genes in the intestine of diabetic rat

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ABSTRACT

Objectives: The aim of this study was to examine the effect of flaxseed on gene expression of intestinal transporters: Niemann-Pick C1 like 1 (NPC1L1), ATP-cassette binding proteins G5 (ABCG5) and G8 (ABCG8).

Methods: Animals were randomly divided into 3 groups 8 rats in each group: group1; normal diet, group2; diabetic rats, and group3; diabetic rats + 4% (w/w) flaxseed. After one-month rats were sacrificed, blood was collected; lipid profiles were determined enzymatically, and mRNA levels were determined by RT-PCR.

Results: Compared to diabetic rats, flaxseed significantly decreased total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides, very low density lipoprotein cholesterol (VLDL-C) and atherogenic index (all $P<0.05$). Intestinal NPC1L1 mRNA was significantly decreased ($P<0.01$) in flaxseed group treatment compared with diabetic animals. Intestinal ABCG5 and ABCG8 mRNAs were significantly increased ($P<0.001$) in flaxseed group treatment compared with diabetic animals.

Conclusion: In conclusion, flaxseed significantly reduced lipid profile and atherogenic index, as compared with the diabetic group. Flaxseed treatment also led to down-regulation of NPC1L1 mRNA and up-regulation of ABCG5 and ABCG8 mRNAs in the intestine of rats.

Keywords: *ABCG5; ABCG8; Cholesterol; Flax; NPC1L1*

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in diabetics. Studies have shown that adults with diabetes have a two to four- fold risk of CVD compared with non-diabetic individuals. Diabetes is also associated with a markedly increased prevalence of dyslipidemia and hypertension. In addition to therapeutic lifestyle changes, medication therapy is available to control some risk factors for cardiovascular disease (CVD) and prevention or treatment of diabetes complications [1, 2]. Knowing genetic regulation of intestinal cholesterol absorption may lead researchers to novel approaches to the treatment and prevention of heart diseases that affect millions of people around the world. Dietary restriction of cholesterol has been used as the primary therapeutic modality treating dyslipidemia. Consequently, studies on the nutritional factors

that can significantly improve dyslipidemia are important [2, 3].

A number of intestinal cholesterol transporters have been known including; scavenger receptor class B member 1 (SRB1) and aminopeptidase N, but the exact role of these transporter is unclear so far. The Niemann-Pick C1 like 1 (NPC1L1) protein, recognized as a vital molecule involved in uptake of cholesterol by enterocytes, as well as ATP-cassette binding proteins G5 (ABCG5) and G8 (ABCG8), known as cholesterol efflux transporters [4]. The ABCG5 and G8 transporters work together and pump plant sterols and cholesterol from enterocytes back into the lumen of intestine for excretion [5]. NPC1L1 protein also may play an important role in the ezetimibe-sensitive (lipid lowering drug) cholesterol absorption pathway in the intestine and may provoke active influx of cholesterol from the lumen into the enterocytes [5]. These data strongly support the opinion that intestinal cholesterol absorption is a multistep process that is regulated by multiple genes and known as target for treatment of dyslipidemia.

Flaxseed is recognized as a rich source of alpha-linolenic acid (ALA), lignans, phytoestrogen, and soluble fiber, all of them have been proposed to reduce plasma cholesterol [6]. Results of a previous experiment suggest that flaxseed decreases both total cholesterol and LDL-C. Flaxseed lignans are also known to show a lipid-lowering effect and cause atherosclerotic plaque regression [7].

We evaluated the effect of flaxseed on lipid profile in diabetic rats and also measured its effect on the expression of NPC1L1, ABCG5 and ABCG8 genes in the rat intestine.

Materials and Methods

Animals

Male Wistar rats with same age and mean body weight of 200-250g were used for this study. The procedure of this experiment was approved by the Animal Research Ethics Committee of Tehran Payame Noor University (Tehran, Iran). After one week of adaptation in animal house under standard hygienic conditions, at 25 ± 1°C and humidity of 55 ± 5% with 12 h light/dark cycle, animals were randomly divided into 3 groups (n=8). Group1: normal diet, group2: diabetic rats, and group3: diabetic rats + 4% of flaxseed (w/w, was added to animals diet).

Diabetes in animals was induced by intraperitoneal injection of 70 mg/kg STZ (STZ dissolved in citrate buffer, pH 4.5) following a fasting period of 24 hours. Rats were maintained for 7 days and then blood glucose levels were measured. Animals with a blood glucose level of more than 300 mg/dl were considered as diabetic [8].

Biochemical analysis

After one month of treatment, animals were anesthetized with diethyl ether and killed by decapitation. Blood samples were collected and centrifuged at 3000g for 10 min at 4 °C and Serum was transferred to a clean small tube and stored at -20°C until analyzed [9].

Total cholesterol (TC), Triglycerides (TG) and high density lipoprotein cholesterol (HDL-C) were measured enzymaticaly according to the manufacture protocols (Pars Azmoon, Iran). Low-density lipoprotein cholesterol (LDL-C), Very low-density lipoprotein Cholesterol levels (VLDL-C) were determined according to the Friedewald equation. Concentration of non-HDL-cholesterol was calculated by subtracting HDL-C from total cholesterol [8, 9]. Atherogenic index was calculated according to the following equation: atherogenic index= total cholesterol/HDL-C

Semi-quantitative RT-PCR

Total RNA from intestine of each rat was extracted with Accuzol Reagent (Bioneer, Korea) according to the manufacturer's protocol. After extraction, synthesis of cDNA was performed using a commercial kit according to the manufacturer's protocol (Fermentas, Lithuania). Semi-quantitative RT-PCR reaction was performed using 2 µl of cDNA solution, 13µl PCR Master Mix (Cinnagen, Iran), 1µl of forward primer, 1µl of reverse primer, and 8µl of deionized water were added into a sterile tube on ice, and then centrifuged for a short time. Thirty five cycles of PCR amplification were performed at the following conditions: denaturation at 95 ºC for 30 s, annealing at 63 ºC for 30 s, and extension at 72 ºC for 30 s using a PCR machine. All reactions were completed with a single additional cycle at 72 ºC for 5 minutes. The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. Band densities were quantified by densitometry using Lab Works analyzing software (UVP, UK). Data are

expressed as the percent ratio of the genes to *β*-Actin. The primers used in this study are presented in Table 1 [9, 10].

Table1. Primers for ABCG5, ABCG8, NPC1L1, and *β*-Actin used in this study

Genes	Primer
ABCG5	Forward :TGCCCT TTCTGAGTCCAGAG
	Reverse**:** GTGCTCTTTCAATGTTCTCCAG
ABCG8	Forward :ATGAGCTGGAAGACGGGCTG
	Reverse: GCCAGTGAGAGCAAGGCTGA
NPC1L1	Forward :GCT TCT TCCGCAAGATATACACTC CC
	Reverse**:**GAGGATGCAGCAATAGCCACATAAGAC
β- Actin	Forward :TGG AAT CCT GTG GCATCCATGAAA C
	Reverse**:** TAAAACGCAGCTCAG TAA CAG TCC G

Statistical analysis

Data were analyzed using one-way ANOVA followed by Tukey post test. Results are expressed as mean ± SD. P-values less than 0.05 was considered as statistically significant.

Results

Effect of flaxseed treatment on blood lipid levels

The results showed that flaxseed significantly reduced total cholesterol ($P<0.01$), triglyceride ($P<0.001$), VLDL-C ($P<0.001$), LDL-C ($P<0.01$) and non-HDL-C ($P<0.01$) in diabetic group (Table 2). Atherogenic index and LDL/HDL ratio were markedly reduced ($P<0.01$ and $P<0.001$ respectively) in flaxseed treatment group compared with diabetic group. The high levels of total cholesterol, triglycerides, and LDL-C were decreased significantly after one month of flaxseed treatment.

mRNA levels

Analysis of reverse transcriptase PCR results showed a significant down-regulation of NPC1L1 mRNA in flaxseed treated rats compared with diabetic group ($P<0.001$) (Fig. 1). ABCG5 and ABCG8 mRNAs were significantly increased ($P<0.001$) in flaxseed group compared with diabetic group (Fig. 2 and Fig. 3).

Table 2. Lipid profiles in different studied groups (n = 8)

Biochemical factors	Flaxseed	Diabetes	Control
TC(mg/dl)	75.10 ± 5.29 [b]	119.40 ± 9.16	71.15 ± 4.03 [b]
TG(mg/dl)	62.93 ± 5.53 [c]	142.17 ± 11.21	64.35 ± 6.82 [c]
LDL-C(mg/dl)	17.15 ± 2.06 [b]	45.89 ± 2.04	16.22 ± 1.08 [b]
HDL-C(mg/dl)	49.70 ± 5.50	45.81 ± 5.31	43.75 ± 5.60
VLDL-C(mg/dl)	12.52 ± 0.35 [c]	28.34 ± 0.61	13.08 ± 0.45 [c]
LDL-C/HDL-C ratio	0.34 ± 0.005 [c]	0.94 ± 0.004	0.36 ± 0.007 [c]
Atherogenic Index	1.51 ± 0.04 [b]	2.6 ± 0.09	1.6 ± 0.08 [b]
Non-HDL-C	25.44 ± 2.67 [b]	73.23 ± 5.17	27.33 ± 3.28 [b]

TC, Total cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein Cholesterol; VLDL-C, Very low-density lipoprotein Cholesterol. Data represent as mean ± SD. [a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$ flaxseed and control compared with diabetic group.

Discussion

Disturbances in cholesterol homeostasis will lead to raised plasma cholesterol levels and increased risk of cardiovascular diseases (CVD), the chief reason of morbidity and mortality in the world. A large number of epidemiological studies have reported a direct link between high levels of plasma cholesterol, specially LDL-C, and risk of CVD [1, 2]. Treatment of conditions causing high levels of plasma cholesterol through intervention in cholesterol synthesis by application of statins has been used for several years. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-regulating enzyme in the pathway of cholesterol biosynthesis, leads to reduced production of VLDL-C particles by the liver, and also upregulation of liver LDL receptor [11, 12]. Both of these routes contribute to reducing plasma LDL-C levels. Nevertheless, a relatively large percent of hypercholesterolaemic patients do not adequately respond to statin therapy or cannot tolerate high doses of statins, consequently these patients my stay at high risk of CVD. As a result other strategies are currently pursued actively, especially those raising HDL-C [13]. In this respect flaxseed has beneficial effects on CVD. In this study flaxseed significantly increased HDL-C levels by 7 %. Epidemiological studies show that every 1mg/dL raise in HDL-C levels reduces CVD risk by 2%-3% [14].

In the diabetic patients, LDL-C is a major risk factor for cardiovascular diseases [12]. In this

study LDL-C was significantly reduced by flaxseed (63%). Studies have shown that the rate of cardiovascular events is reduced by nearly 1% for every 1% decrease in LDL-C [12].

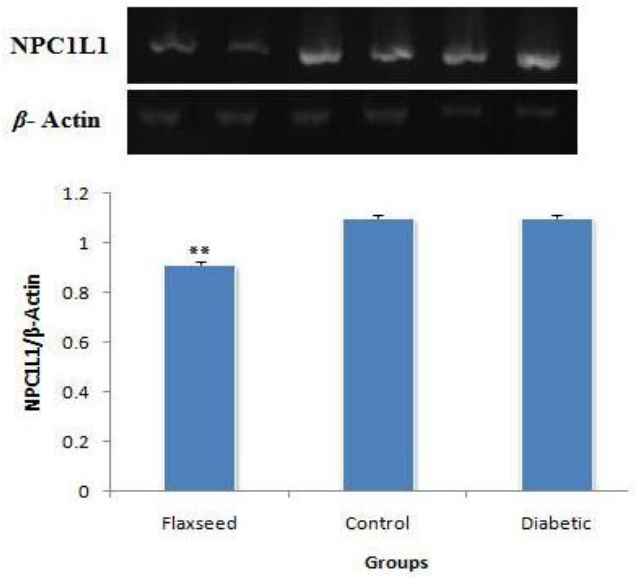

Figure 1. Expression of NPC1L1 mRNA in intestine of flaxseed, diabetic and control rats (n = 8). NPC1L1 mRNA expression significantly reduced in flaxseed group. $^{**}P<0.01$ flaxseed and control compared with diabetic group. Data are presented as mean ± SD.

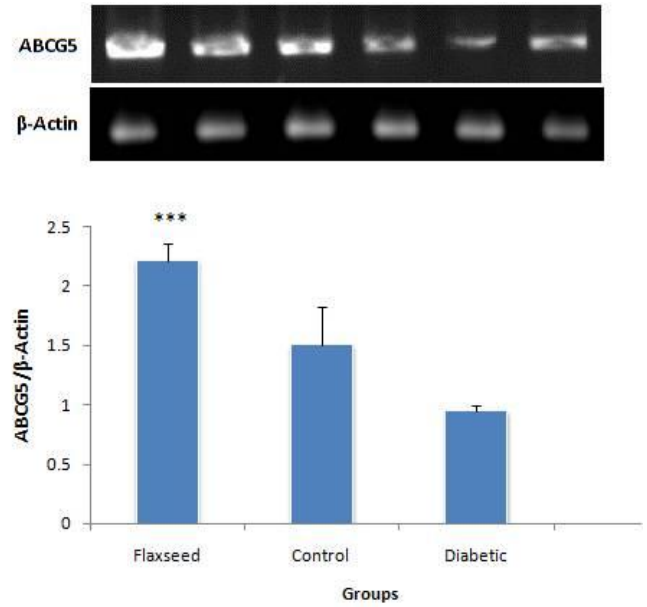

Figure 2. Expression of ABCG5 mRNA in intestine of flaxseed, diabetic and control rats (n = 8). ABCG5 mRNA expression significantly increased in flaxseed group. $^{***}P<0.001$ flaxseed and control compared with diabetic group. Data are presented as mean ± SD.

In this experiment HDL-C levels significantly increased in the flaxseed-treated group. Flaxseed markedly reduced the ratio of LDL/HDL by 61 % in comparison with the diabetic rats. Results of epidemiological and clinical studies have demonstrated that this ratio is an excellent predictor of CHD risk. The levels of non-HDL cholesterol correlate directly with both LDL particle and ApoB numbers. This condition is linked to a higher risk of cardiovascular events in the presence of near-normal LDL-C. In this experiment, non-HDL cholesterol was significantly reduced by flaxseed treatment [15].

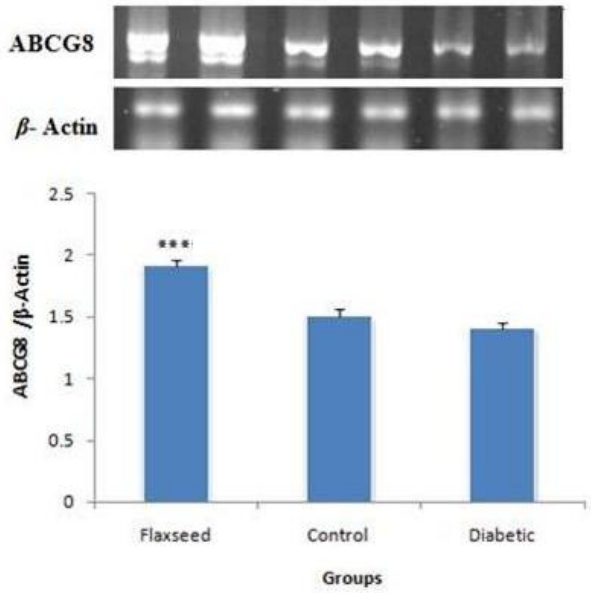

Figure 3. Expression of ABCG8 mRNA in intestine of flaxseed, diabetic and control rats (n = 8). ABCG8 mRNA expression significantly increased in flaxseed group. $^{***}P<0.001$ flaxseed and control compared with diabetic group. Data are presented as mean ± SD.

Cholesterol homeostasis is regulated by a number of ways including: de novo synthesis, intestinal absorption, biliary clearance and fecal excretion. Intestinal absorption has been recognized to be one of the main determinants of plasma lipid profile.

The Liver X receptors (LXRs) are a part of the nuclear receptor family of transcription factors which regulate cholesterol, triglyceride, and glucose metabolism. LXRs have two isoforms including LXRα and LXRβ. Expression of LXRα is restricted to intestine, liver, kidney, adipose tissue, lung, spleen, and macrophages, whereas LXRβ is expressed in almost all tissues. LXR α by controlling expression of a number of genes, such as Niemann-Pick C1 Like 1 (NPC1L1) protein, heterodimers of ATP binding cassette transporters G5 (ABCG5) and G8 (ABCG8) reduces intestinal cholesterol absorption and therefore regulated plasma cholesterol [4].

It has been shown that ABCG5 andABCG8 and the NPC1L1 protein play a critical role in intestinal cholesterol absorption and secretion of cholesterol and non-cholesterol sterols back into

the intestinal lumen for fecal removal. Treatment of mice by Liver X receptor agonist increased excretion of sterols, thus reducing cholesterol absorption. This effect is mediated through ABCG5 and ABCG8. Activation of LXR, increased expression of both ABCG5 and ABCG8 which transferred absorbed cholesterol back to the intestinal lumen [4, 16].

ABCG5 and ABCG8 are expressed mainly in the liver and intestine and are proposed to form a heterodimer for a functional transporter. Deficiency of these genes which occurs in sitosterolemia, is associated with decreasing biliary sterol exertion and increased cholesterol absorption. In the intestinal, ABCG5 and ABCG8 are restricted to the apical membrane of intestinal enterocytes and have shown as LXRα target genes. Activation of ABCG5 and ABCG8 by LXR agonists lead to increased fecal loss of cholesterol, actually these proteins are also recognized as intestinal cholesterol efflux transporters [16]. In this study, treatment with flaxseed significantly increased expression of intestinal ABCG5 and ABCG8 genes in diabetic rats. Results of Linqing Yu, et al (2002) study showed that upregulation of liver and intestinal ABCG5 and ABCG8 leads to a notable rise of biliary cholesterol secretion and hepatic cholesterol synthesis, as well as decreased cholesterol absorption [17].

The NPC1L1 protein has been recently recognized as a vital player in intestinal cholesterol and phytosterol absorption. This protein is expressed in the brush border membrane of small intestine and is necessary for absorption of intestinal cholesterol. Altmann SW, et al. showed that NPC1L1 knock-out animals are resistant to hypercholesterolemic diets and the level of serum lipoproteins and hepatic cholesterol profile is similar to those of wild-type animals which are treated with the cholesterol absorption inhibitor ezetimibe [18].

In this experiment expression of NPC1L1 was significantly reduced in flaxseed-treated group compared with the diabetic group. Cholesterol absorption in NPC1L1 null mouse showed nearly 90% reduction and the remaining residual cholesterol absorption was not sensitive to treatment with ezetimibe [18].

Elevation of plasma cholesterol is related to atherosclerotic coronary heart disease. By reducing plasma cholesterol levels, NPC1L1 inhibition should have useful effects on atherosclerosis [19]. Treatment with flaxseed significantly reduces cholesterol absorption, lowers total cholesterol, and also may inhibit the development and progression of atherosclerosis in diabetic rats.

Conclusion

Since flaxseed is a rich source of alpha-linolenic acid, lignans, phytoestrogen, soluble fiber, all likely antioxidant and hypolipidemic substances, consequently its intake in diabetic rats markedly reduced lipids. On the other hand, flaxseed through reduction of NPC1L1 and raise of ABCG5, and ABCG8 significantly reduced plasma LDL-C and total cholesterol. This research provides the rationale to examine these effects in diabetic patients.

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Conflict of interest

The authors report no conflict of interest.

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