

Co-administration of trientine and flaxseed oil on oxidative stress, serum lipids and heart structure in diabetic rats

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The administration of flaxseed oil or flaxseed oil plus trientine in diabetic rats reduced triglyceride, very low density lipoprotein, and total cholesterol. Furthermore, the combined treatment significantly increased superoxide dismutase activity and attenuated serum Cu²⁺. The results suggest that the administration of flaxseed oil plus trientine is useful in controlling serum lipid abnormalities, oxidative stress, restoring heart structure, and reducing serum Cu²⁺ in diabetic rats.

Keywords: Diabetes, Flaxseed oil, Oxidative stress, Serum lipids, Trientine

Diabetes mellitus (DM) is a complex chronic disorder characterized by hyperglycaemia and disorders in the metabolism of carbohydrates, proteins, lipids, and several essential trace elements¹. DM also causes endothelial destruction and various micro- and macro-vascular complications such as coronary artery disease. This disease is due to deficiency of insulin secretion, insulin action or both². Nowadays, there are many hypoglycemic drugs, such as insulin and sulfonylurea for the treatment of type 2 DM but they have several side effects including insulin resistance, anorexia nervosa, brain atrophy, and liver problems^{3,4}. Therefore, the search for natural compounds with less or no adverse effects from medicinal plants source is warranted because of their ready availability and affordability. These herbal medicines contain biological active substances including antioxidant, hypoglycemic, and hypolipidemic agents.

Flaxseed (*Linum usitatissimum* L.) is full of linolenic acid which is an attractive functional agent for cardiovascular risk reduction⁵. The beneficial effects of eicosapentaenoic acid and docosahexaenoic acid on human health and cardiovascular disease (CVD) are documented⁶. It has been demonstrated that flaxseed or its powder decreases serum lipoprotein⁷, and improves diabetes mellitus in animal

models due to its strong antioxidant activity⁸. Also, the administration of flaxseed oil, flaxseed lignan, or flaxseed decreased serum TNF- α , IL-1 β , IL-6, CRP, and glycosylated haemoglobin concentrations or increased insulin sensitivity in humans⁹⁻¹¹.

Trientine (triethylenetetramine), a Cu²⁺ selective chelator, is currently used for the treatment of heart failure in diabetic rats and humans^{12,13}. Diabetes is accompanied by metabolic changes which lead to tissue-Cu imbalance and accumulation of Cu²⁺ in the extracellular matrix. It has been demonstrated that trientine can stimulate the urinary Cu²⁺ excretion in diabetic rats and patients with type 2 diabetes. Trientine also leads to restoration of cardiac structure and function toward normal. Therefore, trientine can be used a pharmacotherapeutic agent for treating the cardiovascular complications of diabetes¹⁴. In addition, there is no published data on the effects of supplemental flaxseed oil plus trientine on serum biochemical parameters and heart failure in diabetic rats. Therefore, this study has been undertaken to evaluate the protective effect of co-administration of flaxseed oil with trientine on cardiovascular failure and some disorders of metabolic parameters in diabetic rats.

Materials and Methods

Chemicals—Streptozotocin (STZ), trientine, and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma Chemical Co., U.S.A. Superoxide dismutase (SOD) linked immunosorbant assay

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(ELISA) kit obtained from Cayman Chemical Co. (U.S.A). Hemoglobin A_{1c} (HbA_{1c}) kit was purchased from Biosystem Co. (Spain). 2-thiobarbituric acid (TBA), ferric chloride, sodium dodecyl sulfate (SDS) were obtained from Merck (Germany). Flaxseed oil was purchased from Barij Essence Co. (Iran). All the other chemicals were analytical grade.

Experimental design—Male Wistar rats, 200 to 250 g, were purchased from Pasture Institute of Iran (Tehran, Iran). Eight rats in each group were randomly kept in cages under standard laboratory conditions (12 h light/12 h darkness, 21±2 °C). Diabetes was induced by a single intraperitoneal injection (ip) of freshly prepared STZ (60 mg/kg body weight) in ice-cold physiological saline^{15,16}. The animals were considered diabetic, if their blood glucose values were above 300 mg/dL on the 5th day of STZ injection and accompanied by polydipsia and polyuria^{15,16}. Rats were divided into following 5 groups of 8 rats each:

Gr. I: normal control rats, received ice-cold physiological saline (1 mL/kg, ip). Grs II, III, IV, and V received STZ in single dose (60 mg/kg, ip). After 4 weeks of diabetes, Gr. II was considered as untreated control group and received standard pellet chow for 6 weeks. Gr. III was considered as “trientine-treated diabetes group” and received trientine (8–11 mg/day) *via* gavage in aqueous solution once per day for 6 weeks¹³. In group IV rats were fed with a standard pellet diet supplemented with 4% flaxseed oil for 6 weeks¹⁷. In group V rats were fed with a standard pellet diet supplemented with 4% flaxseed oil plus trientine (8–11 mg/day) *via* gavage in aqueous solution once per day for 6 weeks. At the end of the study, fasting blood samples obtained through cardiac puncture for biochemical estimations. The sera were immediately separated from the blood samples by centrifugation and stored at -80 °C until analysis. The experimental protocols were performed with regard to Iranian animal ethics society and local university rules.

Analytical procedures—Serum total cholesterol (TC), triglyceride (TG), glucose (Glu), and high density lipoprotein (HDL-C) were determined by enzymatic method (Pars Azmun kit, Iran) with JENWAY spectrophotometer (model 6105, England). Low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) were calculated with Friedewald formula¹⁸. Serum Cu²⁺ concentrations were determined by atomic absorption (Varian, model spectr AA 240, Australia).

Measurement of serum malondialdehyde (MDA)—The plasma MDA level was assessed as lipid peroxidation by the thiobarbituric acid reactive substances (TBARS) method¹⁹. The serum samples were mixed with thiobarbituric acid solution and incubated for one hour at 95 °C with thiobarbituric acid. The reaction colored product was measured spectrophotometrically at 532 nm using a JENWAY spectrophotometer (model 6105, England). The measurements were done in duplicates and the results were expressed in µM. MDA standards were prepared from 1,1,3,3-tetraethoxypropane.

Ferric reducing/antioxidant power (FRAP) assay—The FRAP assay was performed according to the procedure described by Benzie and Strain²⁰. In this method, the complex between Fe²⁺ and TPTZ gives a blue color with absorbance at 593 nm. FeSO₄·7H₂O was used as a standard of FRAP assay at a concentration range between 100 to 1000 µM.

Histopathological studies—Immediately after sacrifice, the hearts were dissected and fixed in 10% formalin. After processing (dehydrating in gradual ethanol (50 to 100%) and clearing in xylene), the hearts were embedded in paraffin wax and cut into 5 µm thick sections using microtome. The sections were stained with Hematoxylin and Eosin (H&E) stain for studying the histopathological changes²¹.

Measurement of SOD of RBC and Hb A_{1c}—SOD of RBC was determined by ELISA kit (Cayman Chemical Co. U.S.A) with Kayto microplate reader (model RT 21000, China), in accordance with the instructions of the manufacturer. HbA_{1c} was measured by Biosystem kit (Spain), in accordance with the instructions of the manufacturer.

Statistical analysis—Results are expressed as mean ± SD. The data were analyzed by SPSS software (version 17). For statistical analysis of the data, group means were analyzed with one way ANOVA followed by Tukey's test for multiple comparisons. Differences were considered significant at *P* < 0.05 level.

Results

Effect of flaxseed oil and trientine on hyperlipidemia—Table 1 shows the serum levels of TC, TG, LDL-C, VLDL-C, and HDL-C in experimental animals in each group. In untreated diabetic control rats (Gr. II) the serum levels of TC, TG, LDL-C, and VLDL-C significantly increased (*P* < 0.001) compared to normal control rats (group I).

The administration of flaxseed oil or flaxseed oil plus trientine reduced fasting total cholesterol ($P < 0.05$) in groups IV and V by 32 and 37%, respectively compared to group II. However, the administration of trientine alone had no significant effect ($P > 0.05$) on total cholesterol compared to untreated diabetic control rats (Gr. II). Also, the administration of flaxseed oil or flaxseed oil plus trientine caused a 27, 63, and 46% reduction in fasting triglycerides ($P < 0.001$) in groups III, IV, and V respectively compared to group II. On the other hand, treatment of diabetic rats in groups IV, and V with flaxseed oil or flaxseed oil plus trientine resulted in nearly 36% reduction in fasting LDL-C ($P < 0.05$) in groups IV and V compared to groups II and III. In trientine-treated rats (Gr. III), HDL cholesterol increased by 26% ($P < 0.5$; Table 2) compared to untreated diabetic control rats (Gr. II). Likewise, the administration of flaxseed oil or flaxseed oil plus trientine reduced serum VLDL-C ($P < 0.05$) in groups III, IV, and V by 25, 62 and 33%, respectively compared to Gr. II.

Effect of flaxseed oil and trientine on hyperglycemia and the blood HbA_{1c}—Table 2 shows the levels of serum glucose in normal and diabetic animals at the end of the study. The administration of flaxseed oil plus trientine resulted in a significant reduction in serum glucose ($P < 0.05$) in group V compared to groups II, III, and IV. Also, the

administration of trientine and flaxseed oil plus trientine resulted in a significant reduction in HbA_{1c} ($P < 0.05$) in groups III and V compared to groups II and IV, respectively.

Effect of flaxseed oil and trientine on serum Cu²⁺—Table 2 shows the levels of serum Cu²⁺ in experimental groups. In untreated diabetic control rats (Gr. II) the serum level of Cu²⁺ was elevated ($P < 0.001$) compared to normal control rats (Gr. I). The administration of trientine significantly reduced serum Cu²⁺ ($P < 0.05$) in group III compared to other groups. No significant change was observed in serum level Cu²⁺ between groups II and IV ($P > 0.05$). Nevertheless, the feeding of diabetic rats with flaxseed oil plus trientine (Gr. V) attenuated serum Cu²⁺ in comparison to group II.

Effect of flaxseed oil and trientine on MDA and FRAP—Table 2 shows the levels of serum MDA and FRAP in experimental groups. In untreated diabetic control rats (Gr. II), the serum MDA significantly increased ($P < 0.001$) compared to normal control rats (Gr. I). The feeding of diabetic rats with flaxseed oil or flaxseed oil plus trientine (groups IV and V) significantly reduced ($P < 0.001$) MDA levels in serum as compared to the diabetic control rats (Gr. II). However, the supplementation with flaxseed oil or flaxseed oil plus trientine in groups IV and V significantly elevated serum FRAP compared to group II. Moreover, no significant

Table 1—Serum levels of TC, TG, LDL-C, HDL-C, and VLDL-C in experimental groups [Values are mean \pm SD from 8 animals in each group]

Groups	TC (mg/dL)	TG (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	VLDL-C(mg/dL)
I(Control)	53.13 \pm 3.83	99.75 \pm 8.87	17.07 \pm 1.43	39.45 \pm 3.04	19.98 \pm 0.62
II	99.75 \pm 14.31 ^a	138.00 \pm 12.20 ^a	26.37 \pm 1.68 ^a	45.1 \pm 3.04	27.55 \pm 0.46 ^a
III	103.75 \pm 4.62	102.62 \pm 6.43 ^b	26.34 \pm 0.74	57.09 \pm 6.18 ^b	20.52 \pm 0.43 ^b
IV	63.10 \pm 6.84 ^{b,c}	52.62 \pm 6.43 ^{b,c}	16.87 \pm 0.84 ^{b,c}	48.20 \pm 6.21 ^c	10.49 \pm 0.45 ^{b,c}
V	68.87 \pm 2.36 ^{b,c}	78.01 \pm 5.68 ^{b,c,d}	16.82 \pm 0.83 ^{b,c}	48.80 \pm 3.66 ^c	15.60 \pm 0.40 ^{b,c,d}

P values: < 0.001 compared with ^a normal control, ^b diabetic control
< 0.05 compared with ^c Gr.III, ^d Gr.IV

Table 2—Serum levels of Glu, MDA, FRAP, Cu²⁺, and the blood HbA_{1c} in experimental groups [Values are mean \pm SD from 8 animals in each group]

Groups	Glu (mg/dL)	MDA (μ M)	FRAP (μ M)	HbA _{1c} (%)	Cu (μ g/dL)
I(Control)	124.52 \pm 11.23	0.29 \pm 0.03	610.62 \pm 51.06	4.62 \pm 0.15	0.953 \pm 0.013
II	577.59 \pm 13.36 ^a	1.29 \pm 0.05 ^a	501.26 \pm 61.14 ^a	11.71 \pm 0.23 ^a	1.108 \pm 0.008 ^a
III	539.18 \pm 12.24 ^b	1.25 \pm 0.10 ^a	587.69 \pm 45.94 ^b	9.64 \pm 0.19 ^b	0.646 \pm 0.011 ^{ab}
IV	544.45 \pm 24.85	1.10 \pm 0.09 ^{abc}	595.55 \pm 30.06 ^{ab}	11.18 \pm 0.37 ^c	1.121 \pm 0.019 ^c
V	488.47 \pm 34.54 ^{bcd}	0.99 \pm 0.11 ^{abc}	589.25 \pm 59.29	9.37 \pm 0.40 ^{bd}	0.744 \pm 0.023 ^{bcd}

P values: < 0.001 compared with ^a normal control, ^b diabetic control
< 0.05 compared with ^c Gr.III, ^d Gr.IV

difference ($P > 0.05$) was observed for FRAP between groups III and II.

Effect of flaxseed oil and trientine on SOD—SOD activity in RBC is shown in Fig. 1. There was a significant decrease (about 40%, $P < 0.001$) in SOD activity of RBC in group II compared with control rats (Gr. I). The feeding of diabetic rats with trientine or flaxseed oil (groups III and IV) showed no salient difference ($P > 0.05$) compared to group II (untreated diabetic control rats). In group V, however, treatment with flaxseed oil plus trientine significantly increased ($P < 0.05$) SOD activity compared to Gr. II.

Histopathological findings—The histological examination of hearts from the experimental rat groups is shown in Fig. 2. It can be noticed that appearance of the heart from the control group was normal (Fig. 2A). On contrast, diabetes caused extensive damage to myocardial structure with disorganized muscle fibers (Fig. 2B) in the untreated diabetic rats (Gr. II). Oral administration of trientine (Gr. III) led to improve myocardial structure with normalized orientation and appearance (Fig. 2C) compared to diabetes rats without treatment (Gr. II). On the other hand, no significant change in heart ultrastructure was observed in flaxseed oil-treatment group (group IV) in comparison to diabetes rats without treatment (Fig. 2D). However, co-administration of trientine and flaxseed oil plus trientine resulted in a remarkable improvement in myocardial fibers and structure (Fig. 2E) that was relatively comparable to the control group.

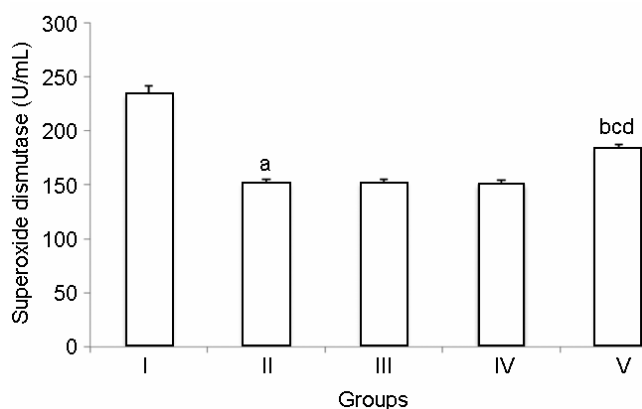


Fig. 1—SOD activity in experimental groups. The data are expressed in mean \pm S.D and $n = 8$ in each group. Normal control (I); diabetic control (II); diabetic rats supplemented with trientine (III); diabetic rats supplemented with 4% flaxseed oil (IV); diabetic rats supplemented with 4% flaxseed oil plus trientine (V) groups.

Discussion

Flaxseed (*Linum usitatissimum* L.) reduces the risk of cardiovascular disease (CVD) due to its alpha-linolenic acid (ALA), which in turn leads to the lowering of triglycerides and blood pressure^{22,23}. On the other hand, it has been demonstrated that trientine-treatment improves cardiomyocyte structure by stimulating the regeneration of cardiac f-actin¹³. In the present study, the administration of flaxseed oil lowered the deranged biochemical parameters especially TC, TG, LDL-C, and VLDL-C in diabetic rats (Table 1). These findings are consistent with published studies about lipid-lowering effects of flaxseed²⁴⁻²⁶. In a previous study, it was demonstrated that the level of MDA elevates in the liver of rats by increasing the liver Cu^{2+} content²⁷. However, in the present study, flaxseed oil supplementation showed a beneficial effect on oxidative stress and it also decreased lipid peroxidation (Table 2). On

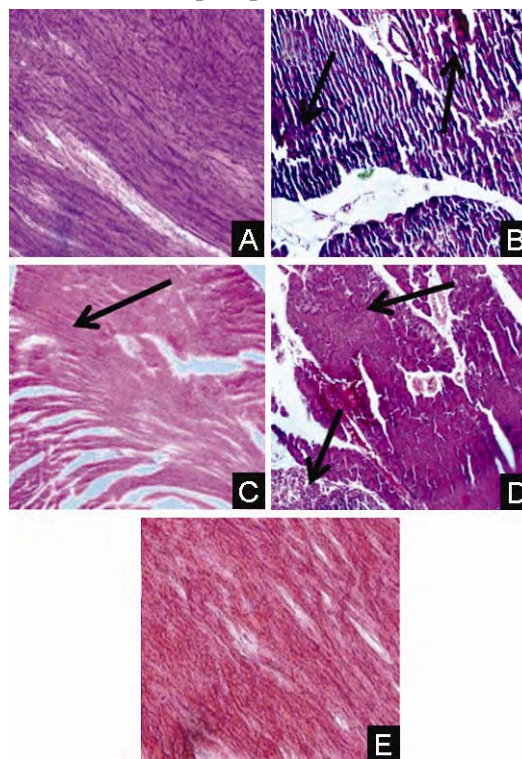


Fig. 2—Photomicrographs of rat heart sections in experimental groups. A, normal rat myocardium (Gr. I); B, untreated diabetic group which shows extensive myocardial damage with diffused lymphocytes and monocytes infiltration plus loss of striations (arrows); C, trientine-treated diabetic group showing the improvement of loss of striations (arrows); D, diabetic group treated with 4% flaxseed oil which shows loss of striations with leucocyte infiltration (arrows); E, diabetic group supplemented with 4% flaxseed oil plus trientine showing normal architecture. (H&E). Figs A, C, and E: 10 \times ; Figs B and D: 40 \times .

the other hand, flaxseed oil increased serum antioxidant capacity in this study (Table 2). Consistent with the present study, there are reports that secoisolariciresinol and secoisolariciresinol diglucoside (SDG) in flaxseed reduce lipid peroxidation by scavenging hydroxyl radical^{28,29}. Also, other studies showed that dietary flaxseed, flaxseed oil, or flaxseed lignan reduce inflammation, oxidative lung damages, lipid peroxidation, or hyperinsulinemia in animals by decreasing reactive oxygen species (ROS)³⁰⁻³². The co-administration of flaxseed oil plus trientine not only decreased the deranged biochemical parameters especially TC, TG, LDL-C, and VLDL-C but also reduced serum Cu^{2+} in diabetic rats (Table 1). However, trientine alone reduced TG, serum glucose, and blood HbA_{1c} , whereas it elevated HDL-C (Tables 1 and 2). Although, flaxseed oil significantly decrease serum HbA_{1c} in humans¹¹, no significant changes were found in HbA_{1c} following flaxseed oil supplementation in this study. However, in the present study, the administration of trientine or flaxseed oil plus trientine significantly reduced HbA_{1c} in treated rats (Table 2). This effect may be due to the effect of trientine on the serum Cu^{2+} metabolism. It has been reported that blood HbA_{1c} in patients with diabetes mellitus is related to the changes in the profile of blood trace elements and the degree of oxidative stress³³. A high correlation between plasma copper and blood HbA_{1c} may show an underlying analogy between diabetic complications and this element³⁴. Although Cu is an essential trace element, free Cu^{2+} ions are highly pro-oxidant in mammalian tissues and may involve tissue damage³⁵. The excess of free Cu^{2+} leads to many pathological conditions such as cardiovascular complications of diabetes due to oxidative damage to membrane lipids and molecules by the generation of reactive oxygen species (ROS)³⁶. Also, there is evidence that Cu^{2+} at high concentrations suppresses extracellular superoxide dismutase (EC-SOD) secretion from cultured vascular smooth muscle cells and this process results in high MDA levels in the liver^{37,38}. On the other hand, the administration of flaxseed oil plus trientine elevated SOD activity. However, neither flaxseed oil nor trientine had positive effect on SOD activity per se (Fig.1).

Numerous studies have showed that chelators provided protection against progression of diabetic complications in animal models of diabetes^{39,40}.

Diabetes, accompanied with increasing cardiac collagen content, disorganized muscle fibers, and diabetes generally leads to heart failure, especially hypertrophy and dysfunction. The use of trientine, however, improves heart failure through the serum Cu^{2+} chelation and it reverses the collagen accumulation in diabetic rat hearts¹³. Also, in this study, the administration of trientine improved cardiac structure in trientine-treated diabetes (Fig. 2B). However, the heart structure in flaxseed oil-treated diabetic rats (Gr. IV) did not differ from control diabetic rats (Gr. II) (Fig. 2C). Likewise, in group IV the serum Cu^{2+} had no significant difference (Table 2) compared to diabetic control group (Gr. II). Therefore, the supplementation of flaxseed oil alone cannot improve cardiac damage structure. The flaxseed oil, generally, reduces the risk of cardiovascular diseases due to decreases in serum lipoprotein⁷. On the other hand, the administration of flaxseed oil plus trientine (Gr. V) restored heart structure, due partly to effect, similar to trientine in the trientine-treated group (Fig. 2C and E). In group V, the serum Cu^{2+} showed a noticeable reduction (Table 2) in comparison to group II (diabetic control group). Therefore, the heart improvement in group V may be due to Cu^{2+} urinary excretion through trientine. Although, flaxseed oil alone had no effect on heart failure regeneration when accompanied with trientine it can reduce plasma lipid abnormalities in diabetic rats.

In the present study, the effects of flaxseed oil plus trientine were not evaluated on inflammatory cytokines, especially tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-1 β . It is suggested that future studies focus on the effects of flaxseed oil plus trientine on inflammatory factors in diabetes.

Conclusion

The co-administration of flaxseed oil plus trientine significantly reduced serum of TG, TC, LDL-C, Glu, and MDA compared with the administration of trientine alone. Therefore, the administration of flaxseed oil plus trientine is useful in the control of serum lipid abnormalities, oxidative stress, restoration of heart structure, and reduction of serum Cu in diabetic rats.

Conflict of Interest

The authors declare that there is no conflict of interest.

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