Original Article

Trigonella foenum-graecum water extract improves insulin sensitivity and stimulates *PPAR* and γ gene expression in high fructose-fed insulin-resistant rats

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Abstract

Background: Insulin resistance is the main defect associated with the metabolic syndrome. In obesity, the decreased adiponectin levels and elevation of plasma-free fatty acids are the main factors associated with insulin resistance. In this study, we evaluated the effect of *trigonella foenum-graecum* (TFG) extract on insulin sensitivity in high fructose-fed insulin-resistant rats.

Materials and Methods: Experimental rats were fed with a high fructose diet for eight weeks. After the first six weeks, the animals were treated with *trigonella foenum-graecum* extract or pioglitazone for two weeks. Serum glucose, triglycerides, cholesterol, and HDL-c were measured. The insulin and adiponectin levels were assayed by the enzyme-linked immunosorbent assay (ELISA), and Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated. The plasma-free fatty acid profile was obtained by gas chromatography. *PPAR*γ and *GLUT4* gene expression were assessed by real-time polymerase chain reaction (PCR) and western blotting.

Results: In the *trigonella foenum-graecum*-extract treated group the following results were obtained: Insulin (49.02 ± 6.93 pmol/L), adiponectin (7.1 ± 0.64 μg/ml), and triglycerides (110.3 ± 16.7 mg/dl), which were significantly different and improved compared to the control group (insulin (137 ± 34 pmol/l), adiponectin (3.9 ± 0.15 μg/ml), glucose (187 ± 15 mg/dl), and triglycerides (217 ± 18 mg/dl). Also the *PPAR*γ gene expression was significantly increased compared to the control group.

Conclusion: This study demonstrates the beneficial effects of *trigonella foenum-graecum* extract on insulin resistance in rats fed on a high-fructose diet. At least three mechanisms are involved, including direct insulin-like effect, increase in adiponectin levels, and PPARγ protein expression.

Key Words: Adiponectin, insulin resistance, peroxisome proliferator-activated receptor gamma, trigonella foenum-graecum, zataria multiflora

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INTRODUCTION

The metabolic syndrome is characterized by insulin resistance, central obesity, hypertension, and dyslipidemia, and increases the risk of cardiovascular disease and type 2 diabetes mellitus.[1] Insulin resistance is the main defect associated with the metabolic syndrome and obesity is the critical

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factor that causes insulin resistance.[2] In obesity, inflammation of the adipose tissue results in increased secretion of free fatty acids. Also, in obesity, adipokine secretion is modified, which can affect the insulin function in the liver and muscle. In insulin resistance, defects in glucose transporter 4 (GLUT4) expression and translocation have been identified in the striated muscle cells and adipose tissue.[3]

The PPARs modulate expression of the genes involved in lipid metabolism.[4,5] The activation of the PPARs stimulates lipid oxidation and lipogenesis. PPARγ activation induces differentiation of the adipocytes and increases insulin sensitivity in mature adipocytes.[6] Hence, synthetic PPARγ ligands, such as, thiazolidinediones (TZDs) are used to control diabetes.[7]

Trigonella foenum-graecum (TFG) also, known as fenugreek, is an annual plant that belongs to the Fabaceae family, which is widely grown in Middle Eastern countries and India.[8,9] The fenugreek seeds are commonly used in the Middle Eastern countries as a spice and in south Asia and Europe, for its anti-diabetic properties. Fenugreek also has a lowering effect on serum thyroglobulin (TG) and total cholesterol concentrations.[10]

In this study, we evaluated the effect of TFG on insulin-resistant rats, to assess the effect of this extract on muscle and hepatic insulin resistance and the possible involved mechanism(s). Skeletal muscle cells are the main sites for insulin function and also for development of insulin resistance.[11,12] The liver is also another insulin-sensitive organ that plays a critical role in energy homeostasis.[13] For these reasons, we investigated *PPARγ* and *GLUT4* gene expression in the liver and muscle tissues.

MATERIALS AND METHODS

Preparation of extract

Trigonella foenum-graecum seeds were obtained from the Department of Pharmacognosy, School of Pharmacy, Kerman University of Medical Sciences, Kerman, I.R. Iran. One-hundred grams of TFG seeds were milled and extracted by the maceration method in 1000 ml of distilled water, at room temperature, for 48 hours. After filtration, the water was evaporated at 40˚C in an oven and the dried extract was stored at –20˚C.

Experimental animals and protocol

Male Wistar rats, weighing 250–300 g, were obtained from the animal house of the Kerman University of Medical Sciences. The rats were housed in an animal room at 22 ± 3°C, with 12 hours of light and 12 hours of darkness. The rats were fed with standard chow and fresh tap water for two weeks. After two weeks, the rats were divided randomly into two groups. One group was fed a 60% fructose diet and the healthy control group (HCG) with standard chow.[14] After six weeks, insulin resistance in the fructose-fed rats was confirmed by oral glucose tolerance (results not shown). The fructose-treated rats were further subdivided into three groups (n = 8):

- The TFG group: This group received an intragastric injection of TFG extract (1000 mg/kg/day)[15]
- The Pioglitazone group (Pio): This group received intragastric injection of pioglitazone (10 mg/kg/day)[16]
- The Diabetic control group (Con): This group was used as an insulin-resistant control group and did not receive any injections.

Treatment was continued for two weeks and the water and food intake were measured daily in this period. Body weight was also measured weekly throughout the eight weeks of treatment.

Blood and tissue collection

At the end of the treatment period, the animals were fasted for 12 hours overnight, blood samples were collected from the heart under ether anesthesia. The blood samples were divided into two vials with or without ethylenediaminetetraacetic acid (EDTA). The vials of 4000 g were centrifuged at 4˚C for 10 minutes. Plasma (for Free Fatty Acid (FFA) analysis) or serum (for other biochemical analysis) was separated immediately. The hind limb skeletal muscle and liver tissues were excised and were immediately frozen in liquid nitrogen and stored at –75˚C, until the experiments were conducted.

Measurement of serum parameters

Serum glucose, triglycerides, cholesterol, and HDL-c concentrations were measured in an RA-1000 autoanalyzer. Blood insulin and adiponectin levels were measured by ELISA using commercial kits (Mouse/Rat Adiponectin or insulin ELISA kit, USCN. China). The HOMA-IR was calculated using the equation: [(insulin (μU/ml) × glucose (mmol/l))/22.5].

Measurement of plasma-free fatty acids

Plasma FFAs were extracted and analyzed by the method explained by Kangani *et al*., with slight modifications.[17]

Briefly, 500 μL of plasma was mixed with 20 μL of pentadecanoic acid (1 mg/ml), as an internal standard. Lipid extraction from the plasma was performed with 2.5 mL of extraction solvent (isopropanol-heptane-

hydrochloric acid (1M)(40:10:1,v/v/v)) containing 0.05 mg butylated hydroxytoluene.

The extracted FFAs were separated by TLC on silica gel plates using a heptane–ether–acetic acid [60:40:3] solvent system. The FFAs were visualized by iodine on the TLC plates and were then scraped and then the FFAs were extracted with chloroform-methanol 3:1. Free fatty acid methyl esters (FAME) were prepared by a reaction with BF3 containing methanol (Sigma). The FFA methyl esters were separated using an Agilent GC-7890A system equipped with a flame ionization detector.

Real-time polymerase chain reaction

Total ribonucleic acid (RNA) from the skeletal muscle (for GLUT4 assay) and liver (for PPARγ assay) tissues was extracted with the RNeasy Mini kit (Qiagen), according to manufacturer's guidelines. The RNA concentration was determined by measuring the absorbance at 260 nm (ND-1000 nanodrop). Then, complementary deoxyribonucleic acid (cDNA) synthesis was performed using the Quantitect Reverse Transcription Kit (Qiagen), according to the manufacture's instructions. Relative Quantitative real time PCR was performed on a rotor-gene Q thermal cycler (Qiagen) using the corresponding QuantiFast SYBR Green PCR kit (Qiagen) according to manufacturer's protocol. The primers that were used in this study are shown in Table 1.

Cycle of threshold (CT) for each sample was determined. ΔCT was calculated via the formula: ΔCT = CT (target gene) - CT (endogenous reference gene (*GAPDH*))

Results were analyzed by the $2^{-\Delta\Delta CT}$ method.

Western blotting

Total protein was extracted from the muscle or liver tissues by homogenization in the radioimmunoprecipitation assay (RIPA) buffer (sigma). The homogenate was centrifuged at 14,000 rpm for 20 minutes and supernatant that contained the proteins was removed. Total protein was estimated by the Bradford method. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were separated by loading 120 µg protein/lane. The proteins were then transferred onto a Polyvinylidene difluoride (PVDF) membrane. Blocking was performed by overnight incubation of the membrane in 5% non-fat skim milk in a Tris-Buffered Saline and Tween 20 (TBST) buffer solutions at 4°C.

The membrane was incubated with appropriate polyclonal primary antibodies (Abcam) (PPARγ or GLUT4 antibody) in a TBST buffer for one hour, and then washed thrice with a TBST buffer (20 minutes each), followed by incubation with anti-rabbit secondary antibody for one hour, at room temperature. Next, the PVDF membrane was incubated with a substrate (western lightening plus ECL, Perkin-Elmer) for one minute. Following this, in a dark room, the PVDF was exposed to Hyblot film (Denvill) for 30 seconds. After development, the band densities were analyzed by the ImageJ software.

Table 1: Primers used in this study

Gene	Primers	PCR product	Accession number
GLUT4	F: ACTGGCGCTTTCACTGAACT	106	NM_012751
	R: CGAGGCCAAGGCTAGATTTTG		
PPARγ	F: CATGCTTGTGAAGGATGCAAG	131	NM_001145367
	R: TTCTGAAACCGACAGTACTGACAT		
GAPDH	F: TGGAGTCTACTGGCGTCTT	138	NM_017008
	R: TGTCATATTTCTCGTGGTTCA		

PCR: Polymerase chain reaction

Statistical analyses

All data are presented as mean ± SEM. Statistical analyses were performed by analysis of variance (ANOVA) and Post-Hoc Tukey test; *P* values less than 0.05 were considered to be significant.

RESULTS

Effect of *trigonella foenum-graecum* on body weight, water, and food intake

There was no significant difference in body weight of the groups at the start of treatment. The body weight showed a significant difference from week eight between the TFG and control groups ($P < 0.0001$). There was also a significant difference between the TFG and pioglitazone groups ($P < 0.05$) and TFG decreased weight gain in comparison to pioglitazone.

There was no significant difference in water or food intake among the TFG, pioglitazone or control groups, however, a significant difference was observed between the control group and the three other groups ($P < 0.0001$) (results shown in Table 2).

Effect of trigonella foenum-graecum on blood glucose, triglycerides, and cholesterol

Blood glucose in the TFG group showed a nonsignificant difference with the control group ($P = 0.34$). TFG significantly decreased the serum triglyceride concentration as compared to the control group ($P = 0.011$). Pioglitazone did not have any effect on the triglycerides, in this study. There was a nonsignificant difference in cholesterol and the LDL-c levels between TFG and the other groups [Table 2].

Table 2: Serum biochemical parameters

Parameter	Groups				*P* value*
	HCG	Con	Pio	TFG	
Initial weight (g)	275±8	272±9	272±12	268.6±7.8	1
Weight after eight weeks (g)	285±9	307±8	294±16	276.1±10.2	0.062
Weight gain (g)	10±2	35±2	22±5	8.5±5.3	0.021
Water intake (ml)	35±0.8	47±2	60±2	45.4±1.9	0.997
Food intake (g)	21.6±0.7	13.4±0.4	13.5±0.3	13.03±0.2	0.999
Insulin (pmol/L)	50±4.8	137±34	40±2.7	49.02±6.93	<0.0001
Adiponectin (μg/ml)	2.9±0.16	3.9±0.15	5.6±0.4	7.1±0.64	<0.0001
Glucose (mg/dl)	132±4	187±15	129±5.8	193±6.8	0. 34
HOMA-IR	2.7±0.37	9.7±2.1	2.1±0.12	3.8±0.6	<0.0001
Cholesterol (mg/dl)	71±12.1	59±3.2	63±4.2	51±3.46	0.914
Triglyceride (mg/dl)	85±13	217±18	200±51	110.3±16.7	0.015
HDL-c (mg/dl)	24.86±1.4	29.25±1.8	29.38±2.3	26.3±1.6	0.962

HCG: Healthy control group, Con: Control, Pio: Pioglitazone, TFG: *Trigonella foenum-graecum* (*n*=8), **P* value demonstrates the significant difference between the TFG and control groups

Effect of *trigonella foenum-graecum* on serum insulin and adiponectin

Compared with the control group, serum insulin was significantly decreased in the TGF group ($P < 0.01$), however, the adiponectin level in the TFG group was significantly increased in comparison with the control group ($P < 0.05$). There was a nonsignificant difference in the insulin and adiponectin levels between the TFG- and pioglitazone-treated groups [Table 2].

Effect of *trigonella foenum-graecum* on the homeostasis model assessment of insulin resistance

There was a significant difference in HOMA-IR between the TFG and the control groups and treatment with TFG decreased the HOMA-IR when compared with the control group ($P = 0.001$). HOMA-IR in the TFG group did not show any difference with pioglitazone treated group [Table 2].

Effect of *trigonella foenum-graecum* on plasma-free fatty acids

There was no significant difference in the total free fatty acids, although the palmitic acid concentration was significantly decreased in the TFG group in comparison with the control group. However, pioglitazone significantly decreased the levels of total free fatty acids, oleic acid, palmitic acid, and palmitoleic acid, as compared with the fructose and TFG groups ($P < 0.05$) [Figure 1 and Table 3].

Effect of *trigonella foenum-graecum* on *GLUT4* and *PPARγ* gene expression

Our results demonstrate that TFG did not have any effect on *PPARγ* and *GLUT4* gene expression on mRNA levels ($P = 0.928$ and $P = 0.995$, respectively). However, pioglitazone significantly increased the mRNA level of PPARγ [Figure 2]. In the HCG group, the mRNA level in both PPARγ and GLUT4 was more than that in the control group.

Table 3: Plasma-free fatty acid levels

Parameter (μmol/L)	Groups				*P* value*
	HCG	Con	Pio	TFG	
Myristic acid	1.07±0.01	1.12±0.06	1.2±0.06	1.20±0.04	1
Palmitic acid	5.9±0.57	11.1±2.6	5.1±0.18	5.87±0.29	0.017
Palmitoleic acid	1.06±0.04	1.4±0.12	1.1±0.01	1.45±0.02	1
Stearic acid	1.3±0.12	2.7±0.12	2.06±0.11	2.00±0.08	0.497
Oleic acid	2.2±0.21	2.9±0.22	1.7±0.08	2.42±0.19	0.966
Total free fatty acids	12.53±1.95	22±2.7	15±2.3	19.44±2.8	1

HCG=Healthy control group, Con=Control, Pio=Pioglitazone, TFG=*Trigonella foenum-graecum* (*n*=8), **P* value demonstrates the significant difference between the TFG and control groups

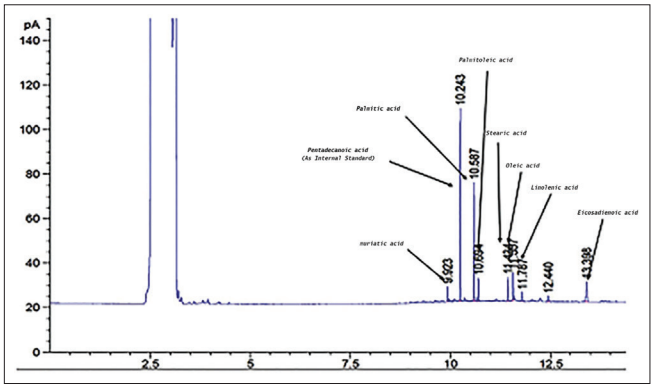

Figure 1: GC chromatogram of free fatty acid in the *trigonella foenum-graecum-treated* group

This study showed that there is a significant difference in the PPARγ protein level between the TFG and the control group. Also, pioglitazone significantly increased the level of the PPARγ protein when compared with the control group. There was no significant difference in the GLUT4 protein level between the groups [Figure 3].

DISCUSSION

In this study, we found that TFG had a beneficial effect on insulin resistance and it increased the insulin

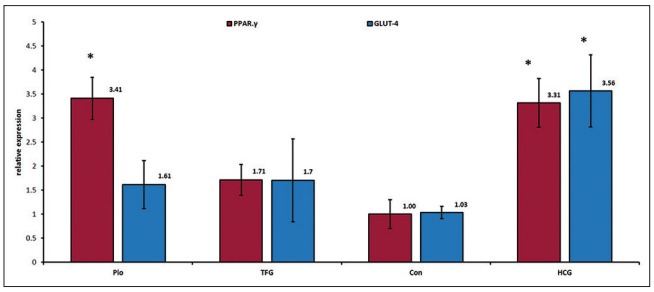

Figure 2: mRNA level of the liver PPARγ and muscle GLUT4. HCG: Healthy control group, Con: Control, Pio: Pioglitazone, TFG: *Trigonella foenum-graecum* ($n = 8$), *Significant difference in the control group ($P < 0.05$)

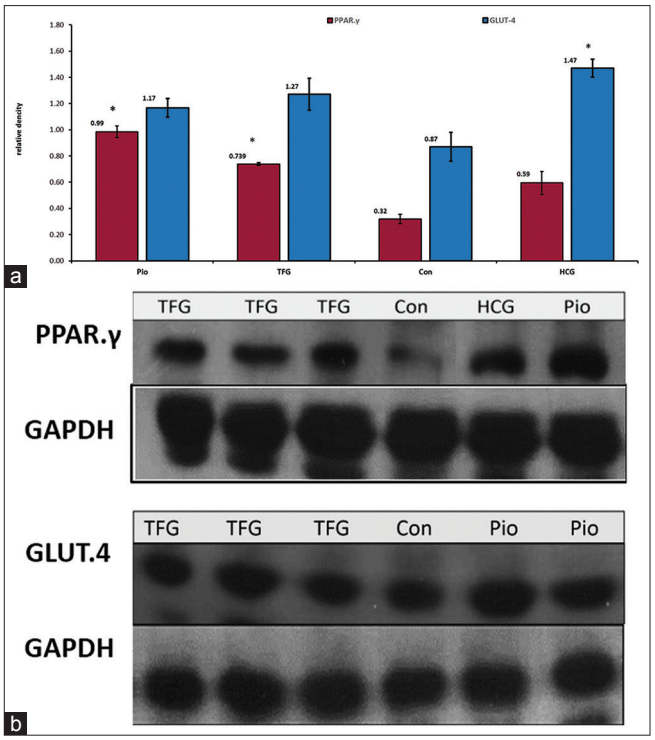

Figure 3: Protein concentration of liver PPARγ and muscle GLUT4 (a and b). HCG: Healthy control group, Con: Control, Pio: Pioglitazone, TFG: Trigonella foenum-graecum ($n = 8$), *Significant difference in the control group ($P < 0.0001$)

sensitivity in fructose-fed insulin-resistant rats. TFG significantly decreased weight gain and triglyceride levels. In Kumar *et al*.'s study, the TFG-seed extract showed antidiabetic effects on alloxan-induced diabetic rats.[18,19] Also, GII, a compound purified from the water-extract of the TFG seeds, reduced blood glucose during the glucose tolerance test (GTT) in sub-diabetic and moderately diabetic rabbits.[20] Premanath *et al*.'s study showed that the ethanol-extract of TFG leaves showed a significant anti-diabetic effect in streptozotocin-induced diabetic rats.[9] These results were confirmed by our study. We did not see any report on the effect of the *trigonella foenum-graecum* extract on *PPARγ* gene expression or the free fatty acid levels.

On the basis of our results, TFG showed a lowering effect on the triglyceride levels in insulin-resistant rats, whereas, pioglitazone showed no significant effect on triglycerides. Therefore, TFG suited the ATPIII guidelines better than pioglitazone, to decrease triglycerides in patients with the metabolic syndrome.[21]

The insulin level was significantly decreased and HOMA-IR increased in insulin-resistant animals treated with the TFG extract. This effect of TFG was comparable with the pioglitazone effect. TFG did not show any effect on total cholesterol, HDL-C or water and food intake.

Our results clearly suggest that TFG improves the insulin sensitivity in the animal model of insulin resistance. Decreased insulin and glucose levels following administration of TFG, suggest that TFG has a direct insulin-like effect,[22] as reduced HOMA-IR is an insulin-resistance indicator, and the glucose level along with a decreased insulin level, implies that the antihyperglycemic effect rises from an insulin-like action rather than an increased insulin secretion.[23]

Trigonella foenum-graecum also significantly increases the adiponectin levels, as seen in our study. A decrease in adiponectin levels, which occurs in obesity, shows a positive correlation with insulin resistance. Weight loss results in a significant elevation of adiponectin levels.[4] Adiponectin receptors, after binding with adiponectin, can mediate the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and also peroxisome proliferator-activated receptors (PPARs), and thereby, increase fatty acid oxidation and glucose uptake. Thiazolidinediones (agonists of PPARs) improve insulin sensitivity by increasing the plasma adiponectin concentration.[4] The adiponectin level is modulated by a number of hormones and factors that participate in the regulation of metabolic processes. Insulin attenuates adiponectin expression in mice and humans.[24] Thiazolidinediones, as mentioned above, increase the expression of adiponectin.[25] *Trigonella foenum-graecum,* may be due to decrease of insulin and increase in the PPARγ protein, increases the serum adiponectin level in insulin-resistant animals. On the other hand, adiponectin inhibits gluconeogenesis in the liver and increases β-oxidation of the free fatty acids in the muscle, and therefore, regulates insulin sensitivity and energy homeostasis.[26] These findings are sufficient to conclude that the antihyperglycemic effect of TFG may be due to the enhancement of adiponectin production.

At the molecular level, TFG increased the PPARγ protein in the liver without increasing the PPARγ mRNA. TFG showed no effect on the GLUT4 protein and mRNA

in the striated muscles. PPAR-γ agonists exert their antidiabetic effect by activation of PPAR-γ, to increase the sensitivity of insulin receptors.[27] Thiazolidinediones significantly increase insulin sensitivity and adiponectin concentration in diabetic patients.[27,28] Yadav *et al*.'s study showed that the PPARγ mRNA increased in the liver of insulin-resistant rats that were treated with rosiglitazone.[29] PPARγ activation also increases fatty acid uptake, adipogenesis, and fat deposition, and improves insulin sensitivity.[7]

As for the role of PPARγ activity in the improvement of insulin resistance, the increasing PPAR-γ protein could be another mechanism for the antihyperglycemic effect of TFG. Also, the decreased serum triglyceride that was induced by TFG, could be correlated with the TG stores in the insulin-sensitive tissues, for PPARγ overexpression.[30]

In diabetes and prediabetes states, the plasma-free fatty acid concentration increases. These findings demonstrate that FFAs may be involved in the complications of diabetes.[31] The developed adipose tissue in obesity is the reason for the increased plasma FFA levels, which leads to inhibition of insulin functions,[11] including inhibition of lipolysis, which in turn further elevates the FFA release into the circulation.[32] The adipose tissue not only releases FFAs, but is also an active metabolic tissue that participates in insulin resistance in the liver and muscles.[33] For these reasons we evaluated free fatty acid concentrations in insulin-resistant rats. However, the total FFA level was not significantly changed with TFG treatment in insulin-resistant rats. The increased FFA level in the blood is an important sign of diabetes and there is an association between increased plasma FFA levels and reduction of glucose catabolism in the skeletal muscle.[7] PPARγ agonists, as mentioned above, enhance the insulin sensitivity in adipose tissue and reduce serum FFA levels.[6] PPARγ agonists insert their insulin sensitizing activity by increasing free fatty acid uptake and storage in adipose tissues.[34]

In conclusion, the findings in this study clearly demonstrated that the *trigonella foenum-graecum* water-extract decreased the level of glucose, insulin, and triglycerides, and improved insulin resistance in the insulin-resistant animal model. This antihyperglycemic effect and increase in insulin sensitivity was applied at least by three possible mechanisms that included, a direct insulin-like effect, increase in adiponectin, and PPARγ protein expression.

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