



Original Research Article

Effect of the Iranian Walnut (*Juglans regia*) Leaves Extract on Gene Expression of Gluconeogenic and Glycogenolytic Enzymes in STZ-Induced Diabetic Rats

**Hossein Khoramdelazad¹, Ahmad pourrashidi¹, Gholamhossein Hasanshahi¹,
Reza Vazirinejad³, Mohammadreza Hajizadeh^{1,2}, Mohammadreza Mirzaei^{1,2},
and Mehdi Mahmoodi^{1,2,*}**

¹Molecular Medicine Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

²Department of Biochemistry, Faculty of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

³Social Determinant of Health Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

*Corresponding author.

Abstract	Keywords
<p>To cure diabetes, there have been growing interests in using hypoglycemic agents from natural products, mainly those derived from plants. In the current survey, hypoglycemic properties of Iranian walnut (<i>Juglans regia</i>) leaves were evaluated by studying mRNA expression levels of the key enzymes involved in carbohydrate metabolism such as, glucokinase (GCK), phosphoenolpyruvate (PEPCK), and glycogen phosphorylase in the liver. Fifty male rats were divided into 5 groups of 10, diabetic groups received 50,100 and 200 mg/kg Iranian walnut leaves extract while diabetic control and normal control received only 0.9% saline for 30 days. Once the experimental period was completed, blood and liver samples were collected. The FBS and insulin levels were measured and analysis of the gene expression was performed by employing Real-time PCR methods. Our findings indicated that the Iranian walnut leaves extract significantly reduces the FBS level in parallel with slight enhancements of insulin in diabetic rat serum. Gene expression analysis showed that Iranian walnut leaves extract increased GCK significantly and inversely decreased gene activity of PEPCK, in diabetic rats. According to this result, it could be suggested that Iranian walnut leaves extract is an effective hypoglycemic agent; the observed effect may possibly be via its ability to enhance insulin secretion and GCK gene expression long with decreasing hepatic glucose output by reducing PEPCK.</p>	<p>Diabetes Glucokinase Glycogen phosphorylase Iranian walnut Phosphoenol pyruvate carboxykinase</p>

Introduction

Diabetes mellitus as a frequent metabolic disorder is characterized by hyperglycemia as a result of failure in insulin production and/or function (Seyed-Mostafa et al., 2012). Investigations in the field of gluconeogenic have highlighted the importance of medicinal plants and antidiabetic with antioxidant properties in diabetic therapy (Rahmatullah et al., 2012).

Liver is an insulin-sensitive organ which plays a fundamental role in the preserving of glucose homeostasis via the regulating process of either glucose utilization or gluconeogenesis (Samoča-Bonet et al., 2012). The liver itself produces glucose in two separate pathways, gluconeogenesis (de novo synthesis of glucose) and glycogenolysis (enzymatic breakdown of glycogen by glycogen phosphorylase catalytic activity) (Chandran, 2015). Within the liver, insulin suppresses transcription of genes encoding gluconeogenic and glycogenolytic enzymes as well as stimulates transcription of genes encoding glycolytic enzyme in order to decrease glucose level (Woitiski et al., 2012). The glucose, which entered in mammalian cells, has to be converted into glucose 6-phosphate by Glucokinase (GCK) as a precursor for further utilization in glycolysis, the pentose phosphate pathway or glycogen synthesis (Haeusler et al., 2015). The expression of GCK is very closely dependent on hepatic insulin level. Hence, GCK (both mRNA and protein) is disappeared from the livers of insulin deficient rats while it was restored following insulin treatment. Also GCK facilitate glucose disposal by the liver and insulin secretion by Langerhans islets.

One other important enzyme involved in metabolism of carbohydrates in mammals, including human being is phosphoenol-pyruvate carboxykinase (PEPCK), which regulate the circulating of glucose level (Stark et al., 2014). PEPCK is a key enzyme that controls gluconeogenesis and glucose output from the liver (Nash et al., 2012). Genes that are involved in gluconeogenic pathway are mainly regulated by insulin (Noguchi, et al., 2013). Additionally, Insulin prevents expression of PEPCK enzymes at the transcriptional level (Granner et al., 2013, Stark et al., 2014). The paramount importance of PEPCK in carbohydrate metabolism in humans is its potential to be employed as a dormant drug target in the treatment of diabetes mellitus (Davidson et al., 2014). Increasing evidences proposing that inhibitors of glycogenolysis

pathway are amongst useful therapeutic target for the treatment of diabetes. Another well-studied enzyme that is regulated by multiple covalent, substrate, and allosteric effectors named glycogen phosphorylase (Martin et al., 1998). The production of glucose from glycogen catalysis to glucose-1-phosphate is rate-limited by this enzyme (Newgard et al., 1989, Sanae et al., 2014).

The aim of diabetes treatment and management is to maintain adaptable circulating glucose level. Concerning the heterogeneity of the diabetes, present therapies are mostly limited. Therefore, to achieve a better therapy protocol, the investigation of new compounds with improved antidiabetic properties is most important and plants are referred to as these targets (Asgary et al., 2008, Celik et al., 2009). Investigations reported that components of green tea have glycemic effects and mimic insulin, and reduces gene expression of the gluconeogenic enzyme PEPCK, and increase Glucokinase mRNA expression in the liver of rats in a dose dependent manner (Li et al., 2011). Thus, the present survey was designed to explore the effects of Iranian walnut (*Juglans regia*) on expression of GCK, glycogen phosphorylase and PEPCK genes in STZ-induced diabetic rats.

Materials and methods

Preparation of hydroalcoholic extract of fresh Iranian walnut leaves was obtained from Raviz (Rafsanjan-Iran). The genus and species of the walnut species were confirmed by expert botanists (Department of Botany, Valiasr University Rafsanjan-Iran). Then, 100 g of fresh walnut leaves was well crushed and 400 ml distilled water/ethanol (25/75) was added. Following 48 h of incubation, the resultant solution was filtered through a filter paper by using a Buchner funnel. The resultant solutions were concentrated by means of a vacuum distillation and decanted dry powder was used to prepare the deserved concentrations (Mard et al., 2010).

Induction of diabetes and Iranian walnut leaves treatments

In this study 50 male albino Wistar rats weighing 180 to 230 g were recruited. Forty rats were injected (intraperitoneal injection) with 45 mg/Kg body weight of streptozotocin (STZ) (diabetic type-1 rats) and ten rats were included as normal group. After being

matched according to body weight, the rats were divided to five groups of 10:

- Group 1: diabetic rats received daily 50 mg/kg Iranian walnut leaves extract (2 ml) for 30 days.
- Group 2: diabetic rats received daily 100 mg/kg Iranian walnut leaves extract (2 ml) for 30 days.
- Group 3: diabetic rats received daily 200 mg/kg Iranian walnut leaves extract (2 ml) for 30 days.
- Group 4: diabetic rats received daily 0.9% saline (2 ml) for 30 days (diabetic control).
- Group 5: normal rats received daily 0.9% saline (2 ml) for 30 days (normal control).

Two milliliters (2 ml) of the above were given to animals *via* gavage syringe. Subsequently animals were housed in the cages and had free access to water and standard food. All of the animal handling processes were performed according to the guidelines of Iranian animal ethics society, Rafsanjan University of Medical Science. At the end of 30 days treatment, blood specimens and liver samples were collected and the levels of FBS and insulin were also measured in all study groups and expression of related genes were analyzed by Real-time PCR.

RNA extraction

To isolate RNA from tissues, rats have been killed humanely, the liver tissues under aseptic situations were removed and immediately frozen in liquid nitrogen. Liver tissue samples were homogenized in TRIZOL™ reagent (Invitrogen) using Mixer 301 and

were subjected to RNA extraction. The purity of extracted RNA was determined by electrophoresis on an ethidium bromide pretreated agarose gel along with measuring absorption at 260/280 nm using spectrophotometric method.

Synthesis of cDNA and quantitative Real-Time PCR

Five micrograms of RNA were reverse transcribed using reverse transcriptase enzyme for 1 h at 37°C for synthesis of cDNA. Quantitative changes of mRNA were assessed by quantitative Real-Time PCR (Bio-Rad CFX) using SYBR-Green detection consisting of SYBR Green PCR Master Mix (Thermo scientific). The sequences of used primer are demonstrated in Table 1. The β -actin was used as housekeeping gene, and each sample was normalized on the basis of its β -actin content. The mRNA encoding target genes were analyzed by employing Real-time PCR method and were normalized by β -actin mRNA (as the housekeeping gene), using the $2^{-\Delta\Delta Ct}$ formula.

Statistical analysis

Results are presented as mean \pm SD. The statistical difference between the means of the various groups were analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple test. For all tests SPSS 18.0 software was used (SPSS Inc., Chicago, IL, USA), and $p \leq 0.05$ was considered statistically significant.

Table 1. Primer sequences.

Transcripts	Primer sequences
Glucokinase (GCK)	F-5' ACTGACTATCCGGCTACATG3 R-5' GATTCCTGCTTGAATAGTGC3
Glycogen phosphorylase	F-5' GTCACCATCACTTCCTTGAAGA3 R-5' GGTGCAGAATCGCGAGTTG3
Phosphoenolpyruvate carboxykinase (PEPCK)	F-5' CCCGAGCACCCAATGACTTTAACC3 R-5' GCGAGTGCGGGATGTGTCA3
B-2 macroglobulin	F-5' TTCTGGTGCTTGTCTCACTGA3 R-5' CAGTATGTTTCGGCTTCCCATTC3

Results

FBS and insulin

Our findings demonstrated that there was a significant difference in the FBS level among all groups and Iranian walnut leaves consumption reduced FBS level

significantly in diabetic treated groups in a dose dependent manner ($p < 0.05$). The FBS concentrations of five groups of rats during the experimental period are shown in Fig. 1.

Analysis of our data demonstrated that the statistically lower insulin levels in diabetic rats in compare to

normal control. Although, extract of Iranian walnut leaves consumption increased insulin level in diabetic rats slightly but this was not significant statistically (Fig. 2).

Fig. 1: The effect of different concentration Iranian walnut leaves on FBS level (mg/dl).

Mean \pm SD; Error bars indicate \pm SD; $p < 0.05$. Group 1: diabetic rats received 50 mg/kg walnut leaves. Group 2: diabetic rats received 100 mg/kg walnut leaves. Group 3: diabetic rats received 200 mg/kg walnut leaves. Group 4: Diabetic rats received 0.9% saline. Group 5: Normal rats received 0.9% saline.

* Significant differences with Group 5 ($p < 0.05$); # Significant differences with Group 4 ($p < 0.05$); \$ Significant differences with Group 2 ($p < 0.05$); & Significant differences with Group 1 ($p < 0.05$);

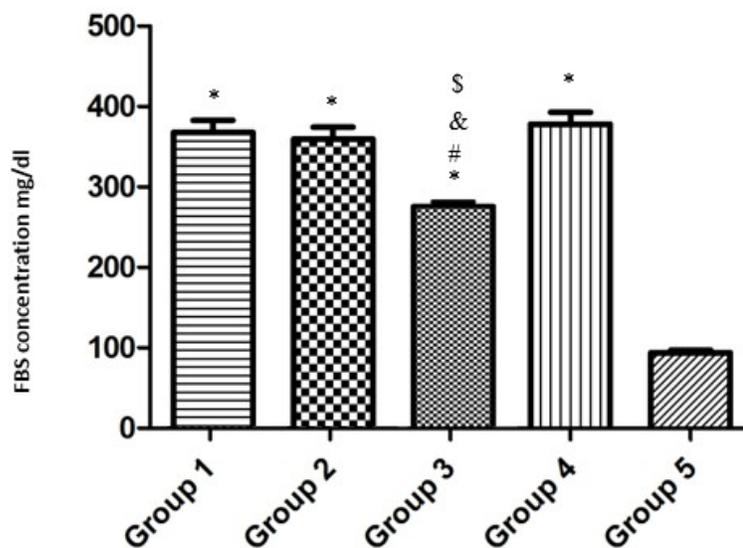
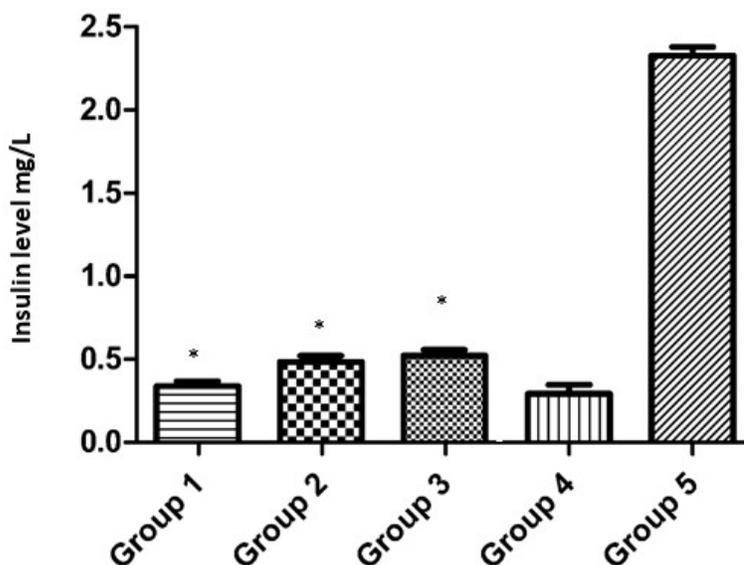


Fig. 2: The effect of different concentration Iranian walnut leaves on insulin level (μ g/l).

Mean \pm SD; Error bars indicate \pm SD; $p < 0.05$. Group 1: diabetic rats received 50 mg/kg walnut leaves. Group 2: diabetic rats received 100 mg/kg walnut leaves. Group 3: diabetic rats received 200 mg/kg walnut leaves. Group 4: Diabetic rats received 0.9% saline. Group 5: Normal rats received 0.9% saline.

* Significant differences with Group 5 ($p < 0.05$);



The mRNA levels of glycolytic and gluconeogenesis enzymes in livers in the normal control group was regarded as 100% expression and the expression in the other groups were accordingly calculated. When compared with control rats, diabetes was found to suppress GCK gene expression in liver (Fig. 3). Using of the Iranian walnut leaves extract caused significant

increased hepatic glucokinase gene expression when compared with control ($p < 0.05$) (Fig. 3). In contrast, PEPCK and glycogen phosphorylase genes were highly expressed in the diabetic rats (Figs. 4 and 5) while, treating with Iranian walnut leaves extract increased glycogen phosphorylase and decreased PEPCK gene expression, however, it was not significant.

Fig. 3: The expressed levels of Glucokinase mRNA (fold) in all groups.

Mean \pm SD; Error bars indicate \pm SD; $p < 0.05$ by ANOVA test; Group 1: diabetic rats received 50 mg/kg walnut leaves. Group 2: diabetic rats received 100 mg/kg walnut leaves. Group 3: diabetic rats received 200 mg/kg walnut leaves. Group 4: Diabetic rats received 0.9% saline. Group 5: Normal rats received 0.9% saline.

* Significant differences with Group 5 ($p < 0.05$);

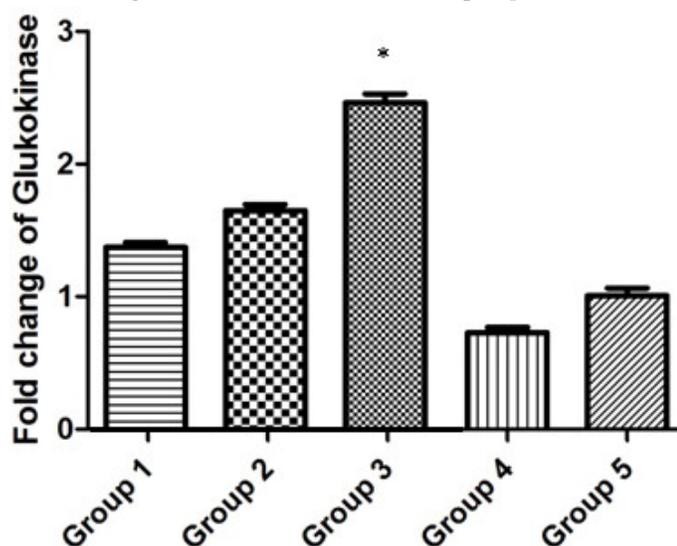


Fig. 4: The expressed levels of phosphoenol pyruvate carboxykinase mRNA (fold) in all groups.

Mean \pm SD; Error bars indicate \pm SD; $p < 0.05$ by ANOVA test; Group 1: diabetic rats received 50 mg/kg walnut leaves. Group 2: diabetic rats received 100 mg/kg walnut leaves. Group 3: diabetic rats received 200 mg/kg walnut leaves. Group 4: Diabetic rats received 0.9% saline. Group 5: Normal rats received 0.9% saline.

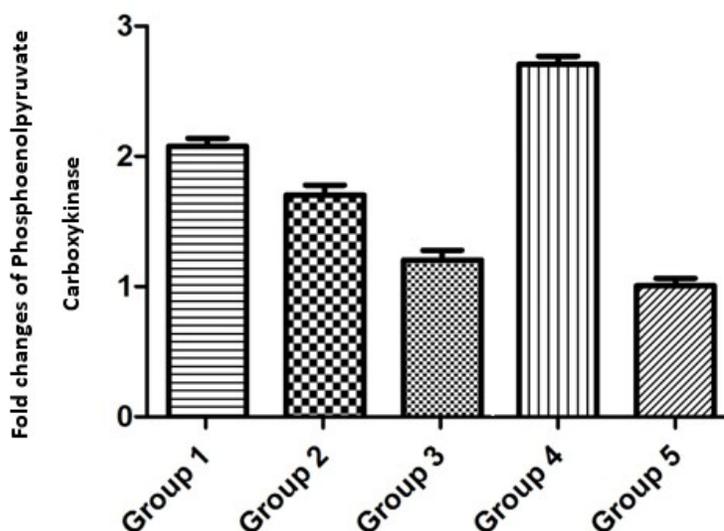
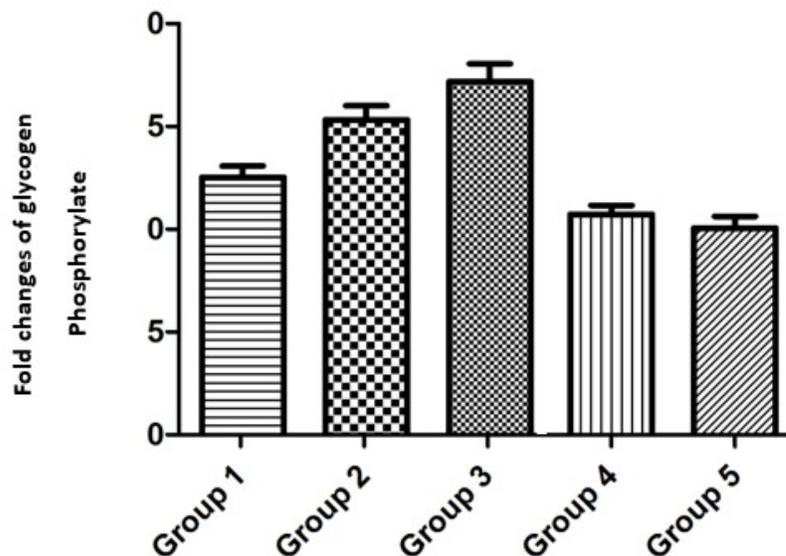


Fig. 5: The expressed levels of glycogen phosphorylase mRNA (fold) in all groups.

Mean \pm SD; Error bars indicate \pm SD; $p < 0.05$ by ANOVA test; Group 1: diabetic rats received 50 mg/kg walnut leaves. Group 2: diabetic rats received 100 mg/kg walnut leaves. Group 3: diabetic rats received 200 mg/kg walnut leaves. Group 4: Diabetic rats received 0.9% saline. Group 5: Normal rats received 0.9% saline.



Discussion

Chronic insulin deficiency and insulin insensitivity are now regarded as two main reasons, leading to either of the decreased hepatic glucose utilization or inversely increased glucose production in diabetes. Insulin indicated to drop the hepatic glucose output by affecting glycogen synthesis and glycolysis as well as inhibiting gluconeogenesis (Jung et al., 2004). Regarding side effects of synthetic chemical drugs, using of hypoglycemic agents from natural products is raised these days. Several bioflavonoids which ubiquitously present in Iranian walnut leaves are reported to improve hyperglycemia in diabetes mellitus (Tiwari and Rao, 2002). In our study Iranian walnut leaves extract significantly reduced FBS while it gently induced serum insulin level. Studies evidenced that the activities of several enzymes such as GCK, and glycogen phosphorylase in the liver of diabetic mice were significantly affected (Newgard et al., 1989, Matte et al., 1997). Zhang et al (2009) claimed that the hepatic activity of glucokinase was decreased by more than 90% in diabetic rats (Zhang et al., 2009). Thus, in present study, to evaluate the antidiabetic mechanism (s) of Iranian walnut leaves extract, the key enzymes of carbohydrate metabolism (e.g. GCK, PEPCK and glycogen phosphorylase) were investigated in the liver of rat at mRNA level using Real-time PCR.

Findings demonstrated that hepatic GCK and glycogen phosphorylase was up regulated, whilst hepatic PEPCK and were remarkably up-regulated in diabetic rats. Due to the fact that serum insulin was increased, it might probably be concluded that the antioxidants (present in walnut leave extract) could simultaneously restore the damaged pancreas and stimulate the secretion of pancreatic insulin (Kaneto et al., 1999). The Iranian walnut leaves have possible potential to accelerate the hepatic glucose metabolism plausible throughout regulating the expression of the functional genes of PEPCK, and GCK. In fact, this hypoglycemic property of Iranian walnut leaves seems to be influenced by notable enhancement of the hepatic GCK mRNA expression. Current results are consistent with previous investigations which reported that rat GCK mRNA expression was increased in Naringin and epigallocatechingallate, a main polyphenolic constituent of green tea (Iynedjian et al., 1988, Nakagawa et al., 2002).

The hepatic GCK has a paramount effect on glucose homeostasis and is a potential target for pharmacological treatment of diabetes, thus, rats over expressing GCK in the liver had reduced blood glucose (Miura, 2014). The elevation of hepatic GCK may lead to increased utilization of the blood glucose for energy production or glycogen storage in the liver

(O'Doherty et al., 1999). A low hepatic GCK activity is also reported to favor the release of glucose synthesized by gluconeogenesis into the circulation. The hepatic PEPCK up-regulation was present in most models of diabetes, and is supposed to contribute to the increased hepatic glucose output seen in this disease. Insulin is the most essential hormone that retards gluconeogenesis. At transcriptional level, insulin down-regulates the mRNAs encoding PEPCK (Celik et al., 2009). In the present study the expression of PEPCK was increased in diabetic rats whereas reduced in Iranian walnut leaves extract treated animals. This is in agreement with Jang et al., which showed that caffeic acid phenethyl ester reduces PEPCK mRNA levels in diabetic rats dramatically (Jang et al., 2008).

Insulin has been demonstrated to inhibit hepatic glucose production throughout stimulation of GCK gene transcription (Ferre et al., 1996). In fact in the present study, changes in hepatic glucose-regulating enzymes could be partly attributed to insulin levels because plasma insulin level was raised in Iranian walnut leaves used by diabetic rats in comparison with the control. Moreover, previous studies indicated that hepatic glycogenolysis processes plays a major role in the regulation of plasma glucose levels in diabetic rats which may propose that glycogen phosphorylase inhibitors might probably be useful in the treatment of diabetes (Jung et al., 2006). Unpredictably Iranian walnut leaves in the current study simultaneously increased both insulin and glycogen phosphorylase activity. Previous studies indicated that insulin inhibits glycogenolysis via stimulation of glycogen phosphorylase activity (Barthel and Schmol, 2003).

Conclusion

Basically, considering the finding of the present study it could be suggested that Iranian walnut leaves having complimentary potency to develop as a hypoglycemic operant in order to treat of diabetes mellitus. Additionally, Iranian walnut leaves also regulate the hepatic glucose metabolism by balancing (up/down-regulating) of the expression of rate-limiting enzymes (GCK, PEPCK and glycogen phosphorylase) in STZ-induced diabetic rats. Enrichment in antioxidant reagents make Iranian walnut leaves as a possible compound for protecting pancreatic islets from STZ-induced damage presumably by eliminating of free radicals as well as repairing the damaged pancreatic

β -cells, and thus, warranties the normal secretion of insulin into serum.

Acknowledgement

This study was financially supported by a grant from Rafsanjan University of Medical Sciences, Iran.

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