Juglans Regia L. Leaf Extract Attenuates Diabetic Nephropathy Progression in Experimental Diabetes: An Immunohistochemical Study

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Abstract

Background: There is accumulating evidence that Juglans regia L. (GRL) leaf extract has hypoglycemic and antioxidative properties. The present study aimed to investigate the protective effects of GRL leaf extract against diabetic nephropathy (DN).

Methods: In total, 28 male adult Sprague-Dawley rats were used. The DN rat model was generated by intraperitoneal injection of a single 55 mg/kg dose of streptozotocin (STZ). A subset of the STZ-induced diabetic rats received intragastric administration of GRL leaf extract (200 mg/kg/day) starting 1 week (preventive group) and 4 weeks (curative group) after the onset of hyperglycemia up to the end of the 8th week, whereas other diabetic rats received only isotonic saline (diabetic group) as the same volume of GRL leaf extract. To evaluate the effects of GRL leaf extract on the diabetic nephropathy, various parameters of apoptosis and inflammation were assessed. Statistical analysis was performed using the SPSS software, version 15.0. The data were compared between the groups using the Tukey’s multiple comparison test and the analysis of the variance. P values <0.05 were considered statistically significant.

Results: Fasting blood sugar (FBS) levels (P=0.001) and histopathological changes in the kidney of diabetic rats attenuated after GRL leaf extract consumption. Greater caspase-3 (P=0.004), COX-2 (P=0.008), PARP (P=0.007), and iNOS (P=0.005) expression could be detected in the STZ-diabetic rats, which were significantly (P=0.009) attenuated after GRL leaf extract consumption. In addition, attenuation of lipid peroxidation in the diabetic rats was detected after GRL consumption (P=0.01).

Conclusion: GRL leaf extract exerts preventive and curative effects against diabetic nephropathy.

What’s Known

• Promoter hyper-methylation of tumor suppressor genes is a common event that occurs in cancer. Since methylation is a reversible modification, agents capable of reversing an abnormal methylation status (e.g. 5-Aza-CdR) should help to combat cancer.
• Evaluation of the epigenetic effect of 5-Aza-CdR on solid tumor has been limited and further investigation is required.

What’s New

• 5-Aza-CdR can limit the proliferation of human pancreatic cancer cell line (PANC-1) through epigenetic reactivation of RASSF1A and consequently up-regulation of Bax in a time- and dose-dependent manner.

Introduction

Nephropathy is one of the most common long-term complications of chronic hyperglycemia. In this regard, several pathophysiological factors account for the development of diabetic nephropathy such as hemodynamic factors, metabolic factors, growth factors/ cytokines, cell signaling and transcription factors, autophagic
activity, inflammation, and oxidative stress. It is well known that among these factors, oxidative stress has a pivotal role not only in the development of diabetic nephropathy but also in its resistance even after good glycemic control, which might be due to accumulated free radicals that are not easily removed. Oxidative stress is generally due to increased polyol pathway activity, autophagic glycosylation, and advanced glycation end-product (AGE) formation after chronic diabetes. Therefore, it has been postulated that the use of antioxidant supplements may offer some protection against this complication through free radical scavenging actions. In the past decades, a large number of natural phenolic compounds and secondary metabolites of plants have been described with antioxidant effects. The seeds, green husks, and leaves of the Persian or common walnut (Juglans regia L.), the best-known member of the Juglans genus, are a rich source of these molecules which have been traditionally used in Iranian folk medicine for the treatment of several diseases such as infections, inflammations, and diabetes and its complications. Flavonoids, phenolic acids, and naphtoquinones are considered as major phenolic compounds in Juglans regia L. leaves. There is accumulating evidence that attributed the beneficial effects of Juglans regia L. leaf extract to a variety of biological activities, including anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-microbial, and anti-fungal properties.

Recently, there have been a few experimental studies on the hypoglycemic effect of Juglans regia L. leaf extract in diabetes mellitus. These studies documented that the administration of Juglans regia L. leaf extract has significantly reduced fast blood sugar (FBS) and HbA1c compared to control groups. Moreover, results of two clinical trial studies have shown that FBG and HbA1c significantly decreased after the consumption of 100 mg Juglans regia L. leaf extract for 3 months and 200 mg Juglans regia L. leaf extract for 2 months compared to placebo groups. An in vitro study also reported that walnut leaf extract inhibits protein tyrosine phosphatase 1B (PTP1B) and enhances glucose uptake. Due to the potent antioxidant and hypoglycemic properties of Juglans regia L. leaf extract, for the first time, we investigated the protective effects of the extract against diabetic nephropathy as a common serious complication of diabetes.

**Materials and Methods**

**Extract Preparation and GC-MS**

Fresh leaves of Juglans regia L. were collected during July-August 2015 from cultivated trees in Khorramabad (Lorestan, Iran) and authenticated by the Natural Resources Research Center of Lorestan Province. Briefly, the leaves were dried, pulverized, and then stored in dark at room temperature. Methanol was added to the pulverized leaves for 72 hours and then filtered through filter paper. The obtained extracts were concentrated at 40 °C. Gas chromatography-mass spectrometry (GC-MS) was carried out using a Hewlett-Packard 6859 with a quadrupole detector, on an HP-5 column, operating at 70 eV ionization energy, using the same temperature program and carrier gas as above. Retention indices were measured by retention times of n-alkanes that were injected after the extract.

**Animals**

In the present experimental study, male adult Sprague-Dawley rats (250-275 g) were obtained from the Laboratory Animal Research Center (Sari, Iran). They were kept in the laboratory under constant temperature (23±2 °C) and 12:12 hour light-dark cycle conditions for at least one week before and throughout the experimental work. All procedures were carried out according to the animal care code of practice of Mazandaran University (IR.MAZUMS.REC.95.S171). The rats had free access to standard chow and drinking water ad libitum throughout the study period.

**Induction of Diabetes and Experimental Design**

Diabetes was induced by a 55 mg/kg single dose of streptozotocin (Santa Cruz Biotechnology, USA) diluted in 0.1 M citrate buffer with PH-4.5. Blood samples were collected from tail vein 48 hours after streptozotocin administration and plasma glucose levels were estimated using a commercial glucometer and test strips (Accu-Chek® Active test meter, USA). Rats with plasma glucose level more than 250 mg/dL were considered as diabetics and included in the study. Juglans regia L. leaf extract was administered by oral gavage (200 mg/kg/day).

The animals were randomly allocated into three groups:

i. Control group (n=7): Received citrate buffer intraperitoneally and isotonic saline orally during 8 weeks.

ii. Diabetic group (n=7): Received single injection of STZ (55 mg/kg) intraperitoneally and given isotonic saline orally during 8 weeks.

iii. Treatment groups (n=14): Received STZ and then orally administered 200-mg/kg/day Juglans regia L. leaf extract from the 1st week (preventive group), from the 5th week (curative group), and continued up to the
end of the 8th week after the induction of diabetes. At the end of week 8, kidneys were removed for histopathological, biochemical, and immunohistochemical assessments. The doses and treatment schedules were based on previous studies and pilot experiments in our laboratory.

**Biochemistry**
The left-side kidneys were thoroughly cleaned of blood, immediately frozen, and stored in a freezer at -80 °C for the assay of tissue malondialdehyde (MDA) levels. The absorbance of the supernatant was measured by a spectrophotometer. MDA levels were expressed as micromoles per milligram of protein.

**Histopathology**
The right-side kidneys were obtained, immediately fixed in 10% buffered formaldehyde, and embedded in paraffin. Five-micrometer serial sections were prepared from the paraffin-embedded blocks using a microtome. For histopathological assessment, some tissue sections were deparaffinized with xylene, stained with periodic acid-Schiff (PAS), and studied by light microscopy. All histological assessments were performed in a blinded fashion.

**Immunohistochemistry**
For immunohistochemistry, some sections were incubated with anti-caspase 3 (1:100; Abcam, Cambridge, USA), anti-COX 2 (1:100; Abcam, Cambridge, USA), anti-PARP (1:100; Abcam, Cambridge, USA), and anti-iNOS (1:100; Abcam, Cambridge, USA) overnight at 4 °C. Then, incubated with secondary antibody conjugated with horseradish peroxidase (Abcam, Cambridge, USA) and detected by DAB for 10 minutes. Afterward, they were dehydrated and mounted. Quantitative analysis was assessed by densitometry using ImageJ software (five immunohistochemical photographs from each sample). Data were expressed as a percentage of the total tissue surface area.

**Statistical Analysis**
Statistical analysis was carried out using the SPSS software, version 15.0 (Chicago, IL, USA). The results were presented as mean±SD. The K-S test was used in order to evaluate the normality of the data. In addition, the Tukey’s multiple comparison test and the analysis of variance were used to compare each two groups and data among the groups, respectively. P<0.05 were considered statistically significant.

**Results**

**GC-MS Analysis**
The principal components identified in Juglans regia L. leaf extract were 2-ß-pinene (17.09%), α-pinene (13.29%), trans-caryophyllene (10.58%), and germacrene D (8.90%). Other minor identified constituents were dl-limonene (3.85%), terpine-4-ol (3.70%), ß-slinene (3.25%), and methyl salicylate (3.07%).

**Blood Glucose Levels**
Fasting blood sugar (FBS) levels for all groups are shown in table 1. Administration of STZ in the diabetic group produced a significant elevation (P=0.001) in FBS level and the hyperglycemia was maintained throughout the experimental period compared to the control group. At the end of the experiment, the FBS levels in the preventive and curative groups were significantly lower compared to the diabetic group (P=0.008), while the differences between the preventive and curative groups were not significant (P=0.09).

**Analysis**
The histogram of the malondialdehyde (MDA) levels for all groups at the end of the experiment is shown in figure 1. Administration of STZ in the diabetic group produced a significant elevation (P=0.01) in MDA level compared to the control group. The MDA levels in the preventive and curative groups were significantly lower compared to the diabetic group (P=0.04), while the differences between the preventive and curative groups were not significant (P=0.08).

**Histopathologic Changes**
Histological examination of the kidney (figure 2) of diabetic rats revealed histopathological changes including, diffused glomerular sclerosis (mesangial expansion)
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and thickening of basement membrane in tubules (A). Furthermore, the kidney of diabetic rats showed somewhat arterial hyalinosis and vascular hypertrophy (A).

Treatment with Juglans regia L. leaf extract in the preventive and curative groups ameliorated the dramatic histological alterations but did not reach the normal structural pattern (B). No detectable injury was shown in the control group (c).

Immunohistochemical Assessment

Figures 3-6 show the immunohistochemical staining of caspase-3, COX-2, PARP, and iNOS, respectively. Administration of STZ in the diabetic group increased the expression of caspase-3 (3A), COX-2 (4A), PARP (5A), and iNOS (6A), while Juglans regia L. leaf extract treatment in the preventive and curative groups reduced the degree of positive staining for caspase-3 (3B), COX-2 (4B), PARP (5B), and iNOS (6B) compared to the diabetic group. The histograms of the quantitative analysis of caspase-3, COX-2, PARP, and iNOS positive staining in the experimental groups are shown in figures 3C, 4C, 5C, and 6C, respectively.

Discussion

The hypoglycemic effects of Juglans regia L. leaf extract in the present study have been previously proven by the experimental study and human clinical trial studies. In this regard, the activity was attributed to the antioxidant capacity of the polyphenols present in walnut leaves, its effects on glucose uptake due to inhibition of protein tyrosine phosphatase 1B, and its effects on beta cell regeneration and its anti-inflammatory properties. One of the most common complications of diabetes mellitus is nephropathy. On the other hand, free radical-induced oxidative stress has been implicated to play an important role in the pathogenesis of diabetic nephropathy. Meanwhile, studies have shown that diabetic nephropathy is a progressive process, despite adequate control of blood sugar, which might be due to the accumulation of reactive oxygen species in tissue. It is well known that hyperglycemia leads to increased glycolysis, which then upregulates reactive oxygen species production and downregulates glutathione production. In this regard, we observed a significant increase in lipid peroxidation, as an index of increased oxidative stress, in the kidney of diabetic rats. Meanwhile, treatment with Juglans regia L. leaf extract significantly ameliorated kidney lipid peroxidation in the preventive and curative groups, while the differences between preventive and curative were not significant. Juglans regia L. leaves contain a large amount of phenolic compounds; well-known free radical scavengers. Phenolic acids, naphthoquinones, and flavonoids are the main phenolic compounds in fresh Juglans regia L. leaves. In this regard, the study of antioxidant activity of Juglans regia L. leaf extract by reducing power assay and scavenging effect on DPPH radicals revealed that walnut leaves cultivars have high antioxidant properties. In vitro study indicated that flavonoids from Juglans regia L. leaves could reduce the reactive oxygen species level in RAW264.7 cells. Carvalho et al. documented that Juglans regia L. leaf extract significantly protected AAPH-induced oxidative hemolysis of human erythrocytes in a time- and
concentration-dependent manner. In another study, the antioxidant potential of ethanolic extract of Juglans regia leaves was measured and the highest ability to chelate Fe$^{2+}$, high reducing power, high antiradical activity, and relatively low prevention of lipid oxidation were documented. Also, an in vitro study of the antioxidant showed that flavonoids in Juglans regia leaf reduced ROS levels in RAW264.7 cells. Results of an in vivo study demonstrated that the administration of walnut leaf extract increased the antioxidant enzymes superoxide dismutase and catalase against CCl4-induced oxidative damage in rat liver. A recent investigation documented that the administration of Juglans regia L. leaf extract resulted in the reversal of biochemical evidence of liver and kidney injury and protection against pancreas in diabetic rats. Several lines of evidence obtained in experimental and clinical studies demonstrated that inflammatory processes play a critical role in the development of the diabetic nephropathy. In this regard, it was well-documented that the enhanced inflammatory response in early
Juglans regia L. leaf extract attenuates criteria of nephropathy in STZ-induced diabetic rats even after the onset of nephropathy, in addition to hypoglycemic effects.

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Conflict of Interest: None declared.

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