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Original Article

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

Davood Nasiry[1], MSc;
Ali Reza Khalatbary[1], PhD;
Hassan Ahmadvand[2,3], PhD;
Fereshteh Talebpour Amiri[1], PhD

[1]Molecular and Cell Biology Research Center, Department of Anatomy, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran;
[2]Department of Biochemistry, Faculty of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran;
[3]Razi Herbal Medicine Research Center, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran

Correspondence:
Ali Reza Khalatbary, PhD;
Department of Anatomical Sciences, School of Medicine, Mazandaran University of Medical Sciences, 20th km of Khazar Abad Road, Sari, Iran
Tel: +98 912 2490583
Fax: +98 11 33543087
Email: khalat90@yahoo.com
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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.

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Keywords ● Nephropathy ● Diabetic ● Juglans regia ● Hyperglycemia ● Antioxidants

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.[1] In this regard, several pathophysiologic 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.[2,3] 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.[3,4] Oxidative stress is generally due to increased polyol pathway activity, autoxidative glycosylation, and advanced glycation end-product (EGE) formation after chronic diabetes.[4] 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.[5,6] Flavonoids, phenolic acids, and naphtoquinones are considered as major phenolic compounds in Juglans regia L. leaves.[7-9] There is accumulating evidence that attributed the beneficial effects of Juglans regia L. leaf extract to a variety of biological activities, including anti-oxidative,[9,10] anti-inflammatory,[11] anti-carcinogenic,[12] anti-microbial,[13] and anti-fungal properties.[14]

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.[15-18] 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[19] and 200 mg Juglans regia L. leaf extract for 2 months[20] compared to placebo groups. An in vitro study also reported that walnut leaf extract inhibits protein tyrosine phosphatase 1B (PTP1B) and enhances glucose uptake.[21] 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.[22]

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 ºC) 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[15,17,23,24] 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.[25] 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 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 glomerural sclerosis (mesangial expansion)

Table 1: Effect of Juglans regia L. (GRL) leaf extract on fasting blood sugar (FBS) level

Groups	FBS (mg/dL) 0d	FBS (mg/dL) 7d	FBS (mg/dL) 28d	FBS (mg/dL) 56d	P value
Control	86.00±8.57	98.60±9.12	94.60±16.43	97.20±15.07	
Diabetic	86.40±8.08	408.00±99.88	442.60±62.03	430.00±70.81*	0.001
Preventive	86.80±8.04	322.00±37.67	369.60±12.46	288.80±41.57**	0.008
Curative	85.20±8.70	373.80±45.35	271.40±67.01	227.80±65.59**	0.008

Data are represented as Mean±SD; *Versus control group; **Versus diabetic group; d: Time after STZ induction

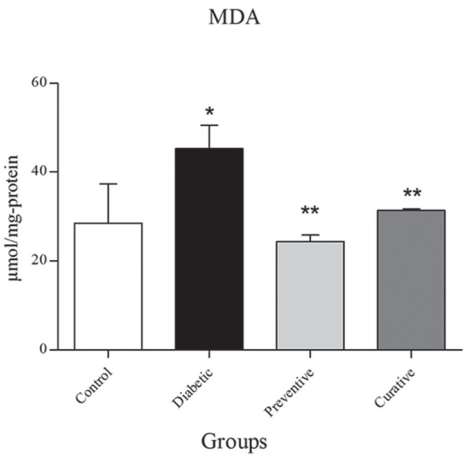

Figure 1: Effects of GRL on MDA level. The histogram shows the levels of malondialdehyde (MDA) at the end of the experiment. Values are expressed as micromole per milligram of protein. *P=0.01 versus control group; **P=0.04 versus diabetic group

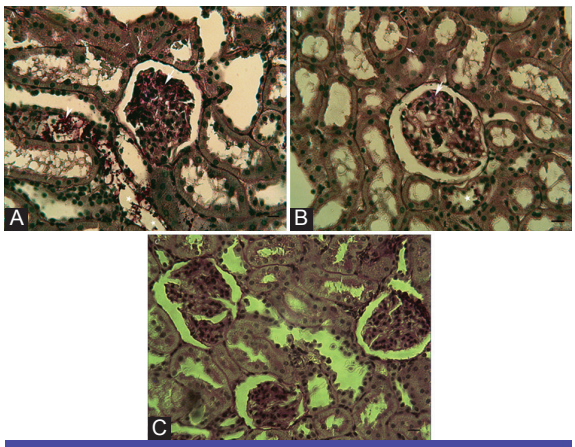

Figure 2: Photomicrographs of the kidney in the diabetic group (A) revealed histopathological changes such as glomerural sclerosis (thick arrow), thickening of basement membrane in tubules (thin arrow), arterial hyalinosis (arrowhead), and vascular hypertrophy (star). Treatment with GRL in the preventive and curative groups ameliorated the dramatic histological alternations (B). No histopathological changes were seen in the control group (C) (stained with PAS; original magnification: ×400, bar: 100 µm).

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 alternations 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[17,18] and human clinical trial studies.[19,20] In this regard, the activity was attributed to the antioxidant capacity of the polyphenols present in walnut leaves,[26] its effects on glucose uptake due to inhibition of protein tyrosine phosphatase 1B,[21] and its effects on beta cell regeneration and its anti-inflammatory properties.[27] 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.[28] 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.[4] It is well known that hyperglycemia leads to increased glycolysis, which then upregulates reactive oxygen species production and downregulates glutathione production.[3] 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.[9,26,29] 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.[8] In vitro study indicated that flavonoids from Juglans regia leaves could reduce the reactive oxygen species level in RAW264.7 cells.[9] Carvalho et al. documented that Juglans regia L. leaf extract significantly protected AAPH-induced oxidative hemolysis of human erythrocytes in a time- and

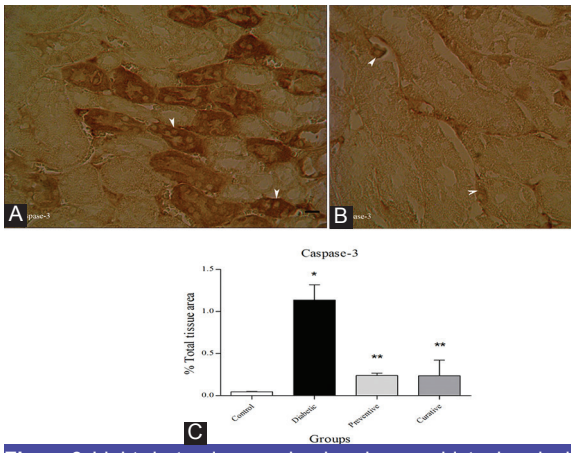

Figure 3: Light photomicrographs show immunohistochemical expression of caspase-3 in the diabetic (A) and treatment (B) groups. The positive staining of caspase-3 is presented with a brown color of cytoplasm (arrowhead) (original magnification: ×400, bar: 100 µm). Densitometry analysis of immunohistochemical photomicrographs for caspase-3 was conducted (C). Data are expressed as a percentage of the total tissue area. *P=0.004 versus the control group; **P=0.009 versus the diabetic group.

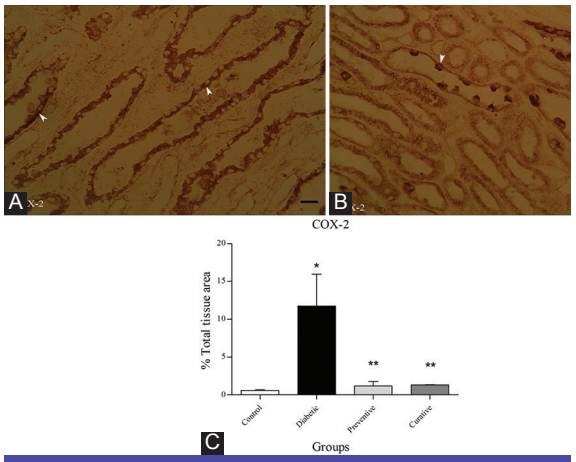

Figure 4: Light photomicrographs show immunohistochemical expression of COX-2 in the diabetic (A) and treatment (B) groups. The positive staining of COX-2 is presented with a brown color of cytoplasm (arrowhead) (original magnification: ×400, bar: 100 µm). Densitometry analysis of immunohistochemical photomicrographs for COX-2 was assessed (C). Data are expressed as a percentage of the total tissue area. *P=0.008 versus the control group; **P=0.009 versus the diabetic group

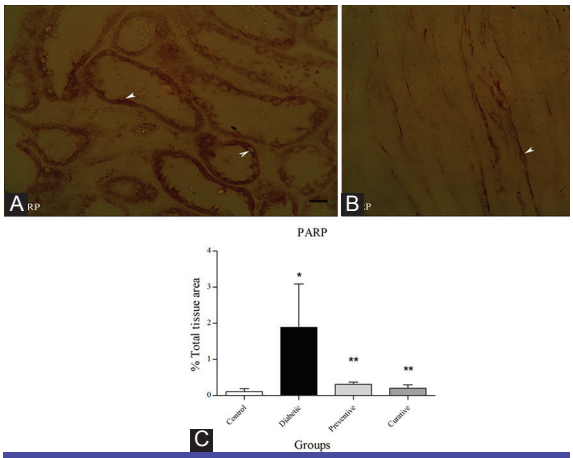

Figure 5: Light photomicrographs show immunohistochemical expression of PARP in the diabetic (A) and treatment (B) groups. The positive staining of PARP is presented with a brown color of cytoplasm (arrowhead) (original magnification: ×400, bar: 100 µm). Densitometry analysis of immunohistochemical photomicrographs for PARP was assessed (C). Data are expressed as a percentage of the total tissue area. *P=0.007 versus the control group; **P=0.009 versus the diabetic group.

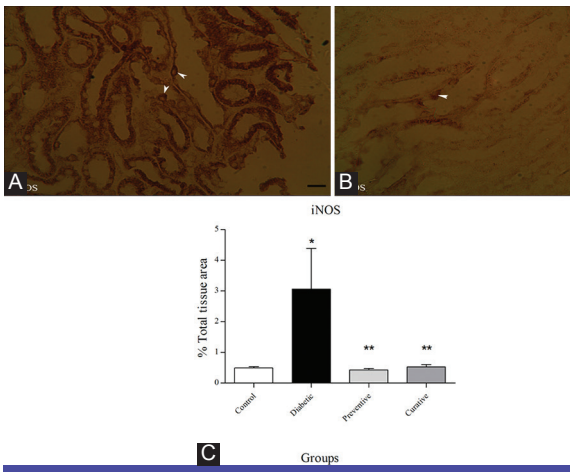

Figure 6: Light photomicrographs show immunohistochemical expression of iNOS in the diabetic (A) and treatment (B) groups. The positive staining of iNOS is presented with a brown color of cytoplasm (arrowhead) (original magnification: ×400, bar: 100 µm). Densitometry analysis of immunohistochemical photomicrographs for iNOS was assessed (C). Data are expressed as a percentage of the total tissue area. *P=0.005 versus the control group; **P=0.009 versus the diabetic group.

concentration-dependent manner.[12] 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.[30] Also, an in vitro study of the antioxidation showed that flavonoids in Juglans regia leaf reduced ROS levels in RAW264.7 cells.[31] 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.[32] 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.[33]

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

diabetic nephropathy was mediated by NF-kB transcription factor; so that selective inhibition of NF-kB reduces the expression of proinflammatory cytokines such as COX-2, TNF□, PARP, iNOS, and IL-1ß.[28,34] Meanwhile, activation of poly (ADP-ribose) polymerase (PARP), a nuclear enzyme which is activated by strand break in DNA, contributes to upregulation on inducible nitric oxide synthase (iNOS) and to nitrosative stress, leading to peroxynitrite formation.[35] Our immunohistochemical assessments showed that increased COX-2, PARP, and iNOS expression in diabetic rats significantly attenuated after treatment with Juglans regia L. leaf extract in the preventive and curative groups, while the differences between preventive and curative were not significant. Hosseinzadeh et al. documented that the aqueous and ethanolic extracts of Juglans regia L. leaves have anti-inflammatory effect against xylene-induced ear swelling in mice, which is mediated by membrane-stabilizing effect that reduces capillary permeability and/or release of inflammatory mediators.[27] In another study, it was shown that Juglans regia L. leaf extract exhibited anti-inflammatory activity against carrageenan-induced hind paw edema model in mice, however, the mechanism underlying this phenomenon is not clear.[11] Polyphenols from Juglans regia L. protected Wistar rats from acute lung inflammation through restoration of glutathione, glutathione reductase, and catalase levels.[36]

Apoptosis is a key mechanism of degenerative diseases, which is triggered by some factors such as hyperglycemia toxicity. Human and experimental studies revealed that hyperglycemia affected the cell survival and induced renal tubular and podocyte apoptosis.[37] These findings were confirmed after administration of a specific inhibitor of caspase-3 which significantly reduced the intensity of apoptosis in diabetic nephropathy.[38] Our immunohistochemical results showed that administration of STZ considerably increased the expression of caspase-3, which plays a critical role in apoptosis. Also, our results showed that this upregulation significantly attenuated after Juglans regia L. leaf extract consumption in the preventive and curative groups, while the differences between preventive and curative were not significant. Javidanpour et al. documented the proliferative effects of Juglans regia L. leaf extract on pancreatic ß-cells in STZ-induced diabetic rats.[15] Results of another study demonstrated that walnut leaf extract has a hepatoprotective effect against carbon tetrachloride-induced cell death.[32] On the contrary, some studies demonstrated that walnut leaf extract showed a higher antiproliferative efficiency than green husk and seed extracts against various cancer cell line such as human renal, oral, breast and colon cancer cell lines,[12] which is more likely related to its phenolic constituents. In addition, it was documented that walnut extracts through modulation of apoptosis-related genes suppressed proliferation and induced apoptosis of cancer cells.[39] Walnuts significantly and dose-dependently reduced apoptosis through decreasing Bax protein levels and inhibition of the caspase-3 activity, while increasing Bcl-2 protein levels against UVB-induced human epidermal keratinocytes apoptosis.[40]

One of the main limitations of this study was the survival time of diabetic animals, especially in the control group that did not receive any treatment. On the other hand, the results of a long-term study are more reliable than a period of 8 weeks.

Conclusion

The main findings of the current study showed that intragastric administration of 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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