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Original article

Rosmarinic Acid Ameliorates Diabetic Nephropathy in Uninephrectomized Diabetic Rats

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Abstract

Objective(s)
Oxidative stress plays an important role in diabetic nephropathy pathogenesis. Rosmarinic acid, a plant phenolic compound, was first used as an antioxidant agent for inhibition of diabetic nephropathy.
Material and Methods
Forty male rats were uninephrectomized from the left flank.The rats were divided in four groups randomly; group one as control, group two diabetic untreatment, groups three and four treatment with rosmarinic acid by 100 or 200 mg/kg/d orally respectively. Diabetes was induced in the second, third and fourth groups by alloxan injection subcutaneously. After 8 weeks treatment, serum malondialdehyde was measured by thiobarbituric acid (TBA) test. Serum creatinine and serum urea were measured by kits. Kidney paraffin sections were prepared and stained by periodic acid Schiff method.Glomerular volume and glomerular number were estimated by stereological rules and glomerular sclerosis was studied semi-quantitatively. Data were analyzed by non-parametric Man Whitney test (using SPSS 13 software) and *P*< 0.05 was considered significant.
Results
Rosmarinic acid (100 or 200 mg/kg) significantly inhibited glomerular hypertrophy, glomerular number loss, glomerulosclerosis, lipid peroxidation, serum urea and creatinine compared with the diabetic untreated group. The level of glomerular number and serum malondialdehyde in the treated groups (100 mg/kg or 200 mg/kg of rosmarinic acid) was maintained at the same level as compared to the control group.
Conclusion
Rosmarinic acid could significantly reduce glomerular hypertrophy, loss of glomerular number, glomerulosclerosis and attenuated serum urea and serum creatinine in diabetic rats.

Keywords: Diabetic nephropathy, Glomerulus, Oxidative stress, Rosmarinic acid

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Introduction

Diabetic nephropathy is the common cause of end stage renal disease (1). Several pathways are thought to be involved in pathogenesis of diabetic nephropathy and its complications, all of them originating from hyperglycemia. Some of these pathways are: increasing and activation of intra-renal rennin angiotensin system (RAS), formation of advanced glycation end products (AGEs), polyol pathway activation, aldol reductase activation, activation of protein kinase C (PKC), increase of some cytokines such as insulin-like growth factor-1 (IGF-1), transforming growth factor beta (TGF-β) and oxidative stress pathway (1, 2).

Oxidative stress means the increase of free radicals because of imbalance between production and neutralization (3). Recently, much attention has been focused on the role of oxidative stress and it has been suggested that oxidative stress may constitute the key and common event in the pathogenesis of different diabetic complications (4).

Oxidative stress can directly induce cell and tissue injuries throughout lipid peroxidation, activation of nuclear factor of Kappa-Beta (NFκB), PARP (poly ADP ribose polymerase) activation, necrosis and induction of apoptosis (5). Oxidative stress activates pathogenic pathways such as RAS, polyol pathway, PKC-B, and AGEs (1- 6).

There is much evidence that pathogenic mechanisms -RAS, AGEs, PKC and some growth factors induce injuries via oxidative stress (1, 3, 5, 7, 8). It may be suggested that oxidative stress acts as a common linking factor in the most important diabetic nephropathy pathogenic pathways. Thus, oxidative stress may constitute a focal point for multiple therapeutic synergies.

Because of increasing demand of patients for the use of natural products and other herbal drugs with anti-diabetic activity, the general trend now is to use the natural products for medicinal application in their natural available form (9).

Rosemary, *Rosmarinus officinalis* L. (Labiatae) is an evergreen perennial shrub grown in many parts of the world. It has been reported to possess a number of therapeutic applications in folk medicines in curing or managing wide range of diseases such as diabetes mellitus, respiratory disorders, stomach problems and inflammatory diseases. Rosemary has long been recognized as having antioxidant molecules, such as rosmarinic acid, carnasol and rosmaridiphenol (10, 11).

In this research rosmarinic acid a phenolic compound was selected for inhibiting diabetic nephropathy because of its beneficial properties including: antioxidant (10-13), anti inflammatory (12-14), reducing NFκB, increasing glutathione transferase, anti Bcl-2 activity (15) and scavenger of peroxynitrite (16). No detailed study has been carried out on the efficacy of rosmarinic acid in modulation of oxidative stress associated with diabetic nephropathy in experimental animals.

Materials and Methods

Forty male mature Sprague–Dawley rats were obtained from Pasteur Institute of Tehran and were allowed to adapt themselves with the new location for one week. This study was approved by the Animal Ethics Committee of the Medical University of Lorestan with accordance to the national health and medical research council guidelines. All animals were uninephrectomized from the left flank for accelerating glomerular hypertrophy and glomerulosclerosis(17). The rats were divided to four groups (10 per each). The studied groups were as follows: group 1 as control, group 2 as diabetic without treatment, 3rd and 4th groups as diabetic treatment with rosmarinic acid in different therapeutic doses.

Rosmarinic acid was prepared from Sigma-Aldrich USA Company (97% C18H16O8 Mw 360,131 –p number 536954).

Diabetes induction

Diabetes was induced after overnight fasting in the second, third and fourth groups by injection of alloxan monohydrate (100 mg/kg) subcutaneously (18). Beta cell degradation by alloxan leads to release of more insulin. Because of acute hypoglycemia, the rats received 10% sucrose solution for 48 hr

instead of drinking water. Five days after induction of diabetes, blood samples were gathered from the end part of tails. Blood glucose was measured by glucometer and the rats with blood glucose level of ≥300 mg/dl (16.7 mmol/l) were considered as diabetic (19). During the first five days after diabetes induction, 1-3 rats per group died because of alloxan toxicity. The rats were kept at 12/12 dark-light period in 21±3 °C temperature. All animals were allowed free access to food and water *ad libitum* during the experiment.

The third and fourth groups were treated with rosmarinic acid 100 or 200 mg/kg/d orally by gavage respectively for eight weeks (20). The rats in the first and second groups received water simultaneously.

The treatment was begun at the first day of diabetes induction. After 8 weeks treatment, animals were anesthetized (Nesdonal 50 mg/kg, i.p.), blood samples were obtained from hearts and allowed to clot for 20 minutes in laboratory temperature and then centrifuged at 10000 rpm for 10 minutes for serum separation (19).

Biochemical study

Serum malondialdehyde (MDA) was measured as the marker of lipid peroxidation by thiobarbitoric acid (TBA) test. Briefly, serum was diluted by buffered saline (1:5) and 400 µl of this mixture was added to 800 µl of trichloroacetic acid (28% w/v) and then centrifuged in 3000 g for 30 min. The precipitation was dissolved in sulfuric acid and 600 µl of this mixture was added to 150 µl thiobarbituric acid (1% w/v) and incubated for 15 min in a boiling water. After incubation, 4 ml n-butanol was added, centrifuged and the absorbtion of red supernatant was recorded spectrophotometrically at 532 nm. The calibration curve was provided by using 1, 1,3,3-Tetraethoxypropan as standard (21). Serum creatinine and urea were determined as markers of kidney function by kits (Ziest Chem. Diagnostic) according to their manufactureres.

Histological study

After blood sampling, the rats were perfused from heart ventricle with saline and then 10% formal saline for 10 minutes under anesthesia.

Then kidneys were decapsulated, weighted and immersed in formal saline solution. After 48 hr fixation, each kidney was cut to slices approximately 1 mm thick. The kidney slices were processed and paraffin sections (5-µm thickness) were prepared. Two sections from each slice were selected (the first and the fifth sections) as section pairs. The sections were stained by periodic acid Schiff (PAS) method.

Stereological study

Glomerular volume per kidney was estimated by point counting rule as follows: sections of kidney slices (one section from each slice) were used. Microscopical field image from each section was projected on a point probe (frame 11×11cm square with 224 + in it that traced on paper) by video projector via a microscope equipped with Leica DFC camera attached to the computer. At total magnification of 80×, points that hit glomerular capillary tufts and mesangium were counted. Glomeruli that fall inside the probe and do not cross the lower and left lines of the probe were selected for point counting. From each kidney, 100-70 glomeruli were assessed (22). Glomerular volume density or volume fraction (Vv) was estimated by the equation 1 (22-23).

Equation 1) $Vv (glom/cortex) = \Sigma Pp / \Sigma Pt$

Σ Pp: sum of points hitting glomeruli and Σ Pt: points in reference space (224. n studied fields).

Total glomerular volume per kidney was estimated by the equation 2 (22).

Equation 2) $Vtotal (glom/kid) = Vv (glom/cortex) . [V kid . Vv (cortex/kid)]$

Kidney weight was used as kidney volume (1 cm³≈1 g) (24). Volume density of cortex per kidney [Vv (cortex/kid)] was also estimated by point counting rule.

Total glomerular number per kidney was estimated by dissector method. From each kidney slice, section pair's 20 µ apart (the first and fifth sections) were used. Microscopic image of the first section (reference) was projected on the dissector frame 1 (square 8×8 cm on paper) and the image of the fifth section (look up) was projected on dissector frame 2 (square 8×8 cm). At total magnification of 80×

in two projection systems, glomeruli were counted if present in frame 1 (not hitting the lower and left lines of frame 1) and absent in frame 2. Numerical density (Nv) of glomeruli per cortex was estimated from the equation 3 (23-25). At least 100 glomeruli per kidney were counted.

Equation 3) NV (glom/cortex)= $\sum Q. M^2 / \sum P .a. d$

$\sum Q$=Number of counted glomeruli, $\sum P$=sum of studied fields from reference sections a= dissector frame area, d= dissector height (section thickness), m= magnification

Total glomerular number per kidney was estimated from the equation 4 (26).

Equation 4) N total (glom/kid)=Nv (glom/cortex). Vcortex

The severity of glomerulosclerosis was studied semi-quantitatively. This part of the study was performed by an experienced histologist in a blinded fashion. Severity in tissue sections was assessed by assigning a score 0 - 4 to each glomerulus according to the tuft demonstrating sclerosis: normal glomerulus=0; up to 25% involvement=1; 25% to 50% involvement=2; 50 to75% involvement =3 and more than 75% involvement =4 (Fig 1-4). The glomeruli were selected for assessment that appeared randomly in microscopic fields. At least 150 glomeruli were assessed in kidney sections of each animal (22). Average glomerulsclerosis score was calculated from total evaluated glomeruli in sections of each kidney and used as an estimation of glomerulosclerosis in each animal.

Statistical analysis: All values are expressed as mean±SEM. The data were compared between groups by Mann-whitney U test.

Statistical analyses were performed using the SPSS 13 for windows software. A *P* value of < 0.05 was considered statistically significant.

Results
Histological results
Effect of rosmarinic acid on glomerular hypertrophy: Our study showed that glomerular volume increased significantly in the untreated diabetic animals in comparison with the control group. Glomerular hypertrophy was significantly inhibited in rosmarinic acid treated groups (100 or 200 mg/kg/d) in comparison with the untreated diabetic group (*P*< 0.05). However, these treatments could not maintain glomerular volume at the same level of glomerular volume in the control group (Table 1).

Effect of rosmarinic acid on glomerular number: Diabetes reduced glomerular numbers per kidney in diabetic untreated group in comparison with the control group significantly (*P*< 0.05). Treatments of diabetic animals with rosmarinic acid (100 or 200 mg/kg/d) significantly inhibited the decrease of glomerular numbers in comparison with diabetic untreated group (*P*< 0.05). Treatments could significantly maintain glomerular numbers at the same level as that of the control group (Table 1).

Effect of rosmarinic acid on glomerulosclerosis: Glomerulosclerosis was significantly inhibited in the treatment groups (rosmarinic acid, 100 or 200 mg/kg/d) in comparison with the diabetic untreated group (*P*< 0.05). These treatments could not significantly maintain glomerulosclerosis at the same level of the control group (Table 1, Figure 1-6).

Table 1. The effect of rosmarinic acid on glomerular volume, glomerular number and glomerulosclerosis in alloxan-induced diabetic rats.

Experimental groups	Glomerular volume/kidney (mm3)	Glomerular number/kidney	Glomerular sclerosis (Score 0-4)
Control	23.76±0.99	28037.71±1630.29	0.195±0.15
Diabetic without treatment	55.9±3.4*	21483.36±1017.39*	1.847±0.18*
Diabetic treated 100 mg/kg RA	30.5±1.7# *	24715.01±1034.11#	0.986±0.048# *
Diabetic treated 200 mg/kg RA	35.53±3.06# *	25638.33±723.27#	0.991±0.043# *

Values are represented as Mean±SEM. RA: Rosmarinic acid. * Significant change in comparison with control at *P*< 0.05. # Significant change in comparison with diabetic without treatment at *P*< 0.05.
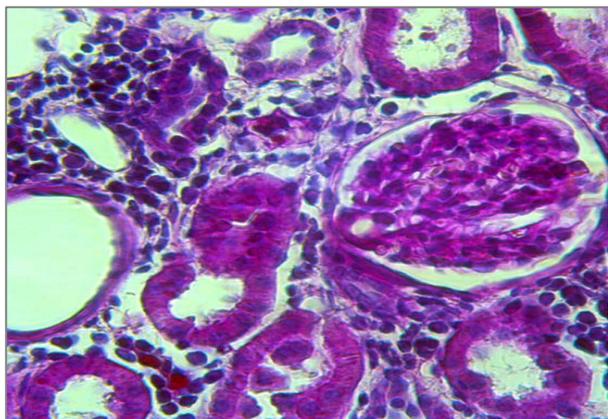
Figure 1. Micrograph from kidney of a diabetic untreated animal demonstrated glomerulusclerosis (Score 4) and lymphocyte infiltration. PAS, 400×.
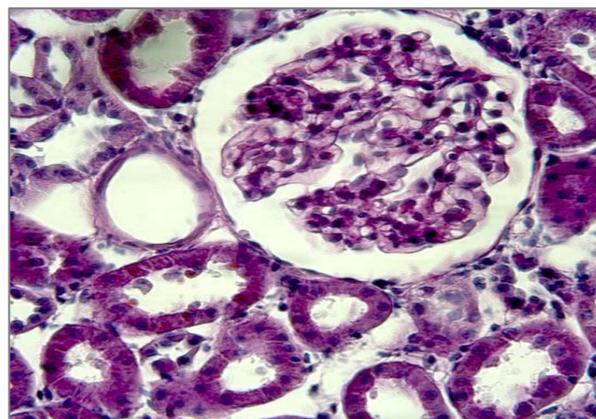

Figure 3. Micrograph from kidney of a diabetic untreated animal demonstrated glomerulusclerosis (Score 2). PAS, 400×.
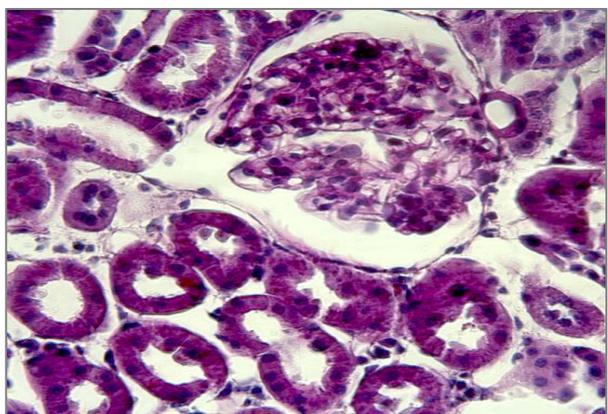

Figure 2. Micrograph from kidney of a diabetic untreated animal demonstrated glomerulusclerosis (Score 3). PAS, 400×.
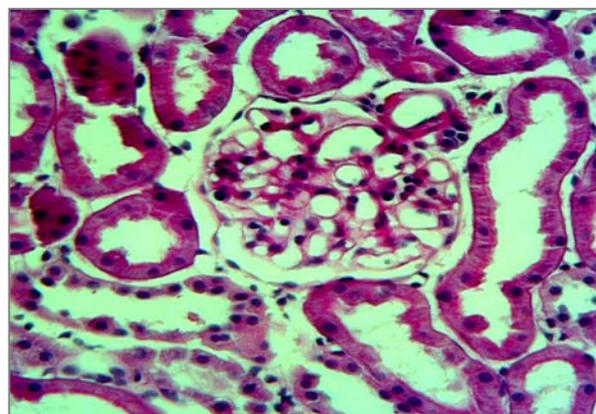

Figure 4. Micrograph from kidney of a diabetic animal treated by rosmarinic acid demonstrated glomerulusclerosis (Score 1) without lymphocyte infiltration. PAS, 400×.

Biochemical results

Effect of rosmarinic acid on serum malodialdehyde

Diabetes significantly increased serum malondialdehyde (MDA) - marker of lipid peroxidation- in comparison with the control group. Treatment of diabetic animals with 100 mg/kg/d or 200 mg/kg/d rosmarinic acid significantly inhibited increase of MDA in comparison with the untreated diabetic animals ($P< 0.05$). These treatments can maintain the level of MDA at the same level compared to that of the control group (Table 2).

Effect of rosmarinic acid on serum creatinine

Serum creatinine as a marker of kidney function significantly ($P< 0.05$) was increased in the untreated diabetic animals in comparison with the control group. Treatment of the diabetic animals with 100 mg/kg/d or 200 mg/kg/d rosmarinic acid could significantly inhibit increase of serum creatinine in comparison with the untreated diabetic animals. Treatment by rosmarinic acid (200 mg/kg/d) could maintain serum creatinine of the treated animal at the same level as that of the control group (Table 2).

Effect of rosmarinic acid on serum urea

Diabetes increased serum urea in the untreated diabetic group in comparison with the non-diabetic control group significantly ($P< 0.05$). Treatment with 100 mg/kg/d or 200 mg/kg/d rosmarinic acid inhibited serum urea in comparison with the untreated diabetic animals significantly ($P< 0.05$). Rosmarinic acid consumption could not maintain serum urea of the treated animals at the same level as that of the control group (Table 2).

Table 2. The effect of rosmarinic acid on serum malondialdehyde (MDA), creatinine and urea in alloxan induced diabetic rats.

Experimental groups	Serum MDA (nmol/ml)	Serum Creatinine (mg/dl)	Serum Urea (mg/dl)
Control	0.51±0.12	0.99±0.45	31.36±1.43
Diabetic without treatment	1.757±020*	1.425±012*	52.04±4.607*
Diabetic treated 100mg/kg RA	0.797±0126#	1.127±0.02# *	38.26±1.81# *
Diabetic treated 200 mg/kg RA	0523±0134#	1.082±0.06#	38.35±2.35# *

Values are represented as Mean±SEM RA:Rosmarinic acid. * Significant change in comparison with control at $P< 0.05$.
 # Significant change in comparison with diabetic without treatment   at $P< 0.05$.

Discussion

This study was conducted on the alloxan-induced diabetic nephropathy rats and describes the renal protective effect of rosmarinic acid. In this study, for the first time the effects of rosmarinic acid were evaluated in the prevention of diabetic nephropathy.

The mechanisms involved in induction and progression of diabetic nephropathy is briefly mentioned in introduction. There is much evidence that oxidative stress plays a key role in the most pathogenic pathways of diabetic injuries. Free radicals such as super oxide can induce cell and tissue injuries through lipid peroxidation, activation of nuclear factor of kappa-beta (NFκB) (5), production of peroxy nitrite, PKC activation and induction of apoptosis. Oxidative stress activates pathogenic pathways such as RAS, polyol pathway, PKC-B and AGEs (1, 6). On other hand, AgII induces superoxide ions formation by activating NADPH oxidase (1, 3, 5). AGEs can induce ROS production and activate PKC by induction of oxidative stress in mesangial cell (7). In fact, in the tangle web of diabetic nephropathy pathogenesis, oxidative stress activates other mechanisms and these mechanisms make injury via oxidative stress and oxidative stress directly leads to injury.

Histological studies

Our study -with designed base stereological methods- showed that the treatment of diabetic animals with rosmarinic acid significantly inhibited glomerular hypertrophy, glomerulosclerosis and loss of glomerular numbers in comparison with the untreated diabetic animals. Rosmarinic acid conserved glomerular numbers at the same level of glomerular number in the control group but could not maintain glomerular volume and glomerulosclerosis at the same level in the control group. Because the nephrons are developed in embryonic period and there is no nephrogenesis after birth, maintaining of glomerular number in diabetes is a very good nephroprotective effect of rosmarinic acid in diabetic nephropathy. Although some researchers reported that consumption of another antioxidant such as vitamin E and alpha tochopherol could decrease glomerular hypertrophy in diabetic animals (27, 28), no stereological methods were used in these studies. Besides, ferulic acid, a polyphenolic antioxidant, can inhibit mesangial hypertrophy and glomerular diameter (29). There are reports that vitamin C, alpha lipoid acid and vitamin E could inhibit or decrease glomerulosclerosis in diabetic rats (30, 32).

Biochemical studies: Our results showed that treatment of diabetic rats by rosmarinic acid could significantly inhibit the increase of serum malondialdehyde, serum creatinine and serum urea in comparisons with untreated diabetic animals. Treatment with rosmarinic acid in two different doses could significantly maintain serum MDA at the level of this variable in control group. Rosmarinic acid could significantly maintain serum creatinine at the same level in the control group only by using 200 mg/kg/day. Inhibition of increased serum MDA in treated diabetic rats is reported with the use of vitamin E (19), probucol (28), alpha lipoic acid (32) and rosemary extract (11). Some

researchers reported inhibition of urea and creatinine in diabetic animal treated by vit E and tocotrienol (19, 33).

Free radicals, especially oxygen-free ones, react with all biological substances; however, the most susceptible ones are polyunsaturated fatty acids. Reaction of free radicals with cell membrane constituents leads to lipid peroxidation. Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors. Any compound with antioxidant property might totally or partially alleviate this damage.

In spite of inhibition of serum MDA (77%-98%) by rosmarinic acid (100 or 200 mg/kg/d), treatment results in inhibition of glomerular hypertrophy (79%-63%) and glomerulosclerosis (52%-52%), maintaining glomerular number (88%-91%), improvement of serum urea (65%-66%) and prevention of serum creatinine increase (68%-79%). Although rosmarinic acid shows a high antioxidant property because of its potent inhibitory effect on lipid peroxidation, it could not maintain glomerular volume, glomerulosclerosis and serum urea at the same level in the control group but had a potent inhibitory effect on serum creatinine increase, and glomerular loss in diabetes.

As diabetes is a multifactorial disease which leads to several complications, it demands a multiple therapeutic approach. We believe that rosmarinic acid with properties such as antioxidant (10-13), anti-inflammatory (12-14), reducing NFκB, increasing glutathione transferase, anti Bcl-2 activity (15) and peroxynitrite scavenger (16), showed good results for pretreatment against diabetic nephropathy. Experimental researches established the role of oxidative stress as a central factor in onset and progression of diabetic nephropathy. In spite of numerous reports and our results that showed efficacy of antioxidative supplements administration on the prevention of diabetic nephropathy, clinical evidences for the effectiveness of antioxidants on the treatment of diabetic nephropathy has not been established and there are several reports that indicated the absence of improvement and even worsening of diabetic nephropathy with antioxidant treatment (29, 34).

Since oxidative stress appears to play an important role as an early etiologic factor in diabetic nephropathy and later progression (29), we suggest antioxidant therapy to the diabetic patients without nephropathy as one of the most important treatment strategies for prevention and inhibition of diabetic nephropathy progression.

On other hand, the early stage diabetic nephropathy is induced by ambient hyperglycemia, but secondary effects are not dependent on persistent hyperglycemia and it is therefore not enough to merely control serum glucose levels in order to retard the development of diabetic nephropathy (35).

In diabetic nephropathy, structural injury develops over years before clinical and laboratory abnormalities such as albuminuria, hypertension, or declining glomerular filtration rate (GFR) appear (36). Thus, waiting for clinical or laboratory manifestation of renal disease before initiating treatment may hinder efforts that prevent progression to end stage of renal disease (19).

Although the detailed molecular protective mechanisms of rosmarinic acid can not be fully explained by our results, our results are satisfactory. Rosmarinic acid -polyphenolic antioxidant - with multi-beneficial properties can be introduced to diabetic patients without diabetic nephropathy for inhibition and progression of diabetic nephropathy.

Conclusion

Rosmarinic acid can inhibit glomerular hypertrophy, save glomerular number, reduce glomerulosclerosis significantly and attenuate serum urea serum creatinine rise in diabetic rats.

Acknowledgment

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