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Original article

Grape seed proanthocyanidin extract ameliorates albuminuria and renal sclerosis in experimental diabetic nephropathy rats

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Background: Grape seed proanthocyanidin extracts (GSPE) prevent and treat a variety of disorders caused by oxidative stress. However, it is unclear whether GSPE has protective effect on rats with diabetic nephropathy.
Objectives: We investigated the effects of GSPE on kidney of diabetic nephropathy rats.
Materials and methods: Thirty male Sprague-Dawley rats were divided into three groups; control, diabetic nephropathy, and diabetic nephropathy, that received GSPE 500 mg/kg for six week. The first, 24 hours urinary albumin excretion (UAE) was studied two weeks after induction of diabetes. Then the UAE was studied each week until the end of the experimental period in all of groups. At the end, the experimental animals were sacrificed and both kidneys were collected and prepared for light microscopy and electron microscopy evaluation and measurement of lipid peroxidation content.
Results: The results showed that the UAE and kidney weight in diabetic nephropathy rats were significantly higher than in controls. Increases of the mesangial matrix, glomerular basement membrane thickness, broadening of foot process, and lipid peroxidation content in diabetic nephropathy group rats were observed. In addition, apoptosis cells were present in this group when compared to controls. These features were reversed when the GSPE was administered to the experimental rats.
Conclusion: GSPE prevented the progression of diabetic nephropathy in rats by its antioxidant properties

Keywords: Apoptosis, diabetic nephropathy, glomerular basement membrane, GSPE, lipid peroxidation

Diabetic nephropathy (DN) is a main cause of end stage kidney disease and imposes financial and social problem to communities [1-3]. DN is characterized functionally by protein and albumin excretion in the urine and structurally by glomerular hypertrophy, mesangial matrix accumulation, and kidney interstitial tissue fibrosis. These changes are related to the loss of renal function. Research suggests that oxidative stress plays a major role in the pathogenesis of diabetic nephropathy. In addition, antioxidant has been suggested to have potentially helpful effects in the human kidney and experimental diabetes [4, 5]. Oxidative stress has been reported to play role as a common mediator in apoptosis [6, 7] and increased in diabetic nephropathy [4]. Recent evidences suggest that hyperglycemia can increase apoptosis in vitro. It also can induce cellular damage in diabetes in vitro [8].

Grape seed proanthocyanidin extracts (GSPE), have been reported to possess a variety of powerful properties such as anti-oxidant, anti-in□ammation, radical-scavenging, and renal protecting anti-tumor activity. It was reported that GSPE had protective effect on the kidneys of diabetic rats [9-11, 12]. Therefore, we investigated the effects of the GSPE on apoptosis, oxidative stress, and the functional as well as pathologic changes in the diabetic nephropathy rats.

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Materials and methods

Thirty male Sprague-Dawley rats (140 to 180g) were prepared from animal house center of Ahwaz Jondishapur University of Medical Science. All animals were housed in cages with 12/12 hours light/dark cycle at 21±2°C. All experimental animals were carried out in accordance with Ahwaz University Ethical Committee.

Grape seed proanthocyanidin extracts (GSPE) (95% purity) were purchased from Hangzhou Joymore Technology Co., Ltd (China). The other chemicals and reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, USA).

Induction of diabetes

The animals were fasted for 24 hours prior to the induction of diabetes. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (50mg/kg body weight) freshly dissolve in citrate buffer (O.1M pH 4.5), while control rats were injected with vehicle buffer only. Blood samples were obtained from the tail vein of the animals at 72 hours after STZ injection and fasting blood glucose levels were determined with a glucose strip test in a glucometer (Easy gluco blood glucose monitoring system, Infopia, Korea). Rats with fasting blood glucose levels above 250 mg/dl were used as the diabetic animals.

Experimental design

Thirty rats were used and were divided into three groups of 10 rats each. The groups were divided as follows; control group, untreated diabetic nephropathy group, and treated diabetic nephropathy group (GSPE, 500mg/kg body weight). The GSPE was given in normal saline solution by oral gavages for six week from the beginning of fifth week after the induction of diabetes, since we found a significant increase in urinary albumin excretion rate in diabetic rats in comparison with control rats at the end of fourth week. Rats were kept individually in metabolic cage with access to drinking water for measurement of twenty-four-hour urinary albumin, two weeks after the induction of diabetes, then each week until the end of the experimental period. Urinary albumin excretion was measured by quantitative reaction with bromocresol-green [13].

At the end of the experimental, six rats selected randomly from each group were sacrificed under ether anesthesia. The right kidneys were collected and perfused (to be free of blood) with PBS and were fixed separately for light microscopy and electron microscopy evaluation. In addition, the left kidney was dissected and rinsed with isotonic saline and then blotted dry and weighed. After weighing, kidney tissue was minced and a homogenate was prepared with 5% (w/v) potassium phosphate buffer (0.1 M, pH 7.4) using a homogenizer (HeidolphSilentcrosher M, Germany). Homogenate was then centrifuged at 16000 × g for 20 minutes to remove nuclei and cell debris. This supernatant was used for the measurement of lipid peroxidation.

Renal histology

Renal cortical tissue samples were fixed in 10% paraformaldehyde, dehydrated through increasing concentrations of ethanol, and embedded in paraffin. Then two sections of 5□m thickness (an interval of 100 μm) per animal were stained with periodic acid-Schiff (PAS) techniques. Renal cortex was evaluated for extracellular matrix deposition.

TUNEL staining

Intranucleosomal DNA fragmentation was labeled in situ using an In Situ Cell Death Detection Kit (Roche, Germany). Paraf□n-embedded tissue 5 μm-thick sections were mounted on slides. After deparaffinization and dehydration, sections were digested with proteinase K and treated according to the protocol provided with the kit. Labeled nucleotides were catalytically added to 3'-OH ends of DNA by terminal deoxynucletidyl transferase (TdT) in a template-independent manner (TUNEL-reaction). Sections were then reacted with anti-fluorescein antibody conjugated with horseradish peroxidase as a reporter enzyme. Diaminobenzidine (DAB) for the In Situ Cell Death Detection Kit was used as the chromogenic substrate for peroxidase, producing a brown reaction product that marked the nuclei of apoptotic cells. Sections were counterstained with hematoxylin. Apoptosis positive cells were evaluated by light microscope.

Electron microscopy

Tissue samples obtained for electron microscopy were fixed with 3% glutaraldehyde in phosphate buffer, and then post-fixed in 1% aqueous osmium tetroxide. Following dehydration in increasing concentrations of ethanol, tissues were embedded in epon resin. Ultrathin sections were prepared and were double stained with uranyl acetate saturated in 70% ethanol and lead

citrate respectively. The ultrathin sections were evaluated under a CM 10 Philips transmission electron microscope.

Estimation of lipid peroxidation

Degree of lipid peroxidation in kidney tissue homogenate of all the experimental animals was determined for thiobarbituric acid reactive substances (TBARS) formation [14]. Five hundred micro liter of supernatant was mixed with 1.5 ml TCA (10%). After centrifugation (4000 × g for 10 minutes), 1.5 ml of supernatant was added to 2 ml TBA (0.67%) and heated for 30 minutes at 100°C. After cooling, the sample was extracted with 2 ml of n-butanol. After centrifugation at 4000 × g for 15 minutes the organic phase was collected and the absorbance was read spectrophotometrically at 535 nm using a blank containing all the reagents except the tissue homogenate. Values were expressed as nmol/mg protein. As 99% of the TBARS is malondialdehyde (MDA), TBARS concentrations of the samples were calculated from a standard curve using 1,1,3,3-tetramethoxypropane.

Protein determination

Protein was measured by the method of Bradford [15] using bovine serum albumin as standard.

Statistical analysis

Data are expressed as the mean□SE. One-way ANOVA by SPSS for Windows (version 18) was used for statistical analysis followed by Tukey's t-test. P <0.05 was assumed as statistically significant.

Results

Effects of GSPE on kidney weight

In the diabetic nephropathy group, kidney weight increased significantly compared to control rats **(Figure 1)**. After administration of GSPE, a significant decrease of kidney weight was found in treated diabetic nephropathy group in comparison to diabetic nephropathy group.

Effects of GSPE on 24h Albumin Excretion in Urine

Figure 2 shows a significant increase of 24 hours albumin excretion in urine of the diabetic nephropathy group in comparison to the control rats. In the diabetic nephropathy group that received GSPE, a decrease in 24 hours albumin excretion in urine was observed in comparison to untreated diabetic nephropathy group. These differences were statistically significant.

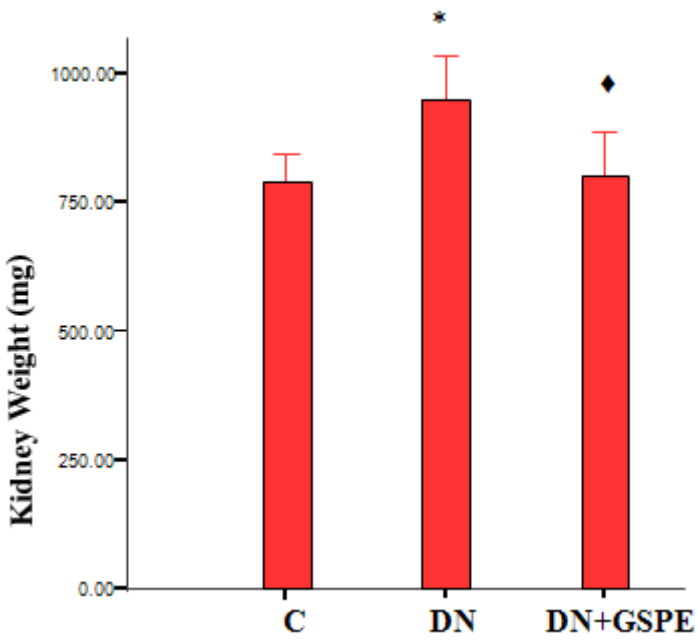

Figure 1. Kidney weight changes between groups, control (C), diabetic nephropathy (DN), diabetic nephropathy treated with GSPE (DN+GSPE). $^{*}p$ <0.05 as compared with control, $^{f\&}p$ <0.05 as compared with diabetic nephropathy.

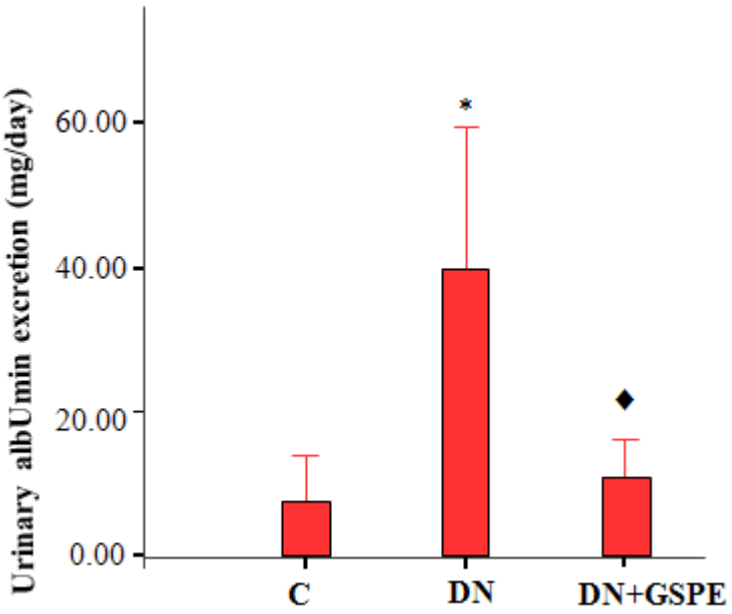

Figure 2. Changes 24 hours albumin excretion (UAE) during the experimental period between different groups. $^{*}p$ <0.05 as compared with control, $^{f\&}p$ <0.05 as compared with diabetic nephropathy.

Effects of GSPE on renal histology of diabetic nephropathy rats

A moderate increase of the mesangial matrix in the glomeruli of most diabetic nephropathy rats in comparison to the control rats is shown in **Figure 3**. This was evident by an increase in PAS-positive mesangial matrix area. Therapy with GSPE for six weeks reversed the mesangial matrix accumulation found in by diabetic nephropathy. The glomerulus contained less PAS-positive matrix material and the capillary loops were more widely open following GSPE therapy.

Effects of GSPE on apoptosis in diabetic nephropathy rats

Figure 4 shows the apoptosis occurred in the tubular epithelial cells and glomerular cells in diabetic nephropathy group. We did not observe apoptotic cells in tubular and glomerular cells in control rats. The apoptotic cells decreased in GSPE- treated diabetic nephropathy group when compared to untreated diabetic nephropathy rats.

Effects of GSPE on ultrastructure of glomeruli in diabetic nephropathy

The thin glomerular basement membrane (GBM) with relatively spread filtration slit pores are shown **Figure 5**. In contrast, in diabetic nephropathy rats, we observed a thicker GBM than in control rats. In addition, we observed irregularity in podocyte foot process dimensions and broadening of foot process in diabetic nephropathy rats. These effects were less evident by treatment with GSPE.

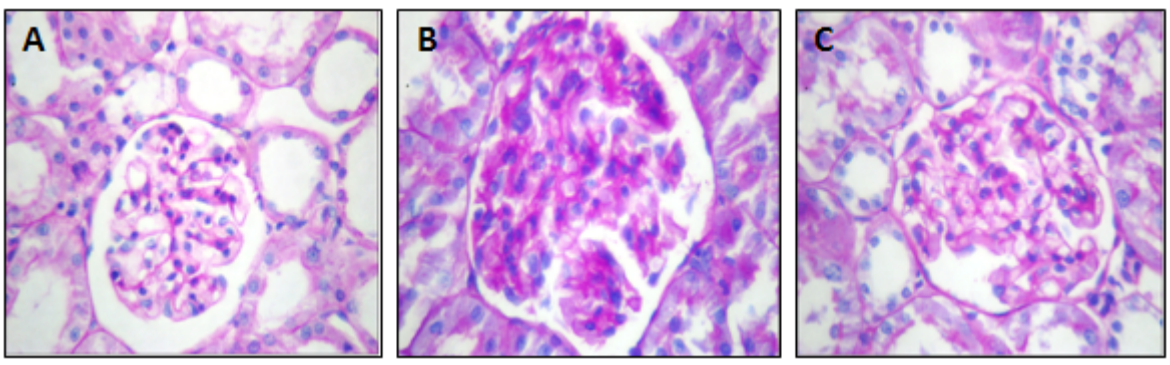

Figure 3. Light microscopy of renal tissue. **A:** PAS staining of glomeruli from a control rat. **B:** a diabetic nephropathy rat, **C:** a diabetic nephropathy rat that was administrated GSPE. Magnification ×400

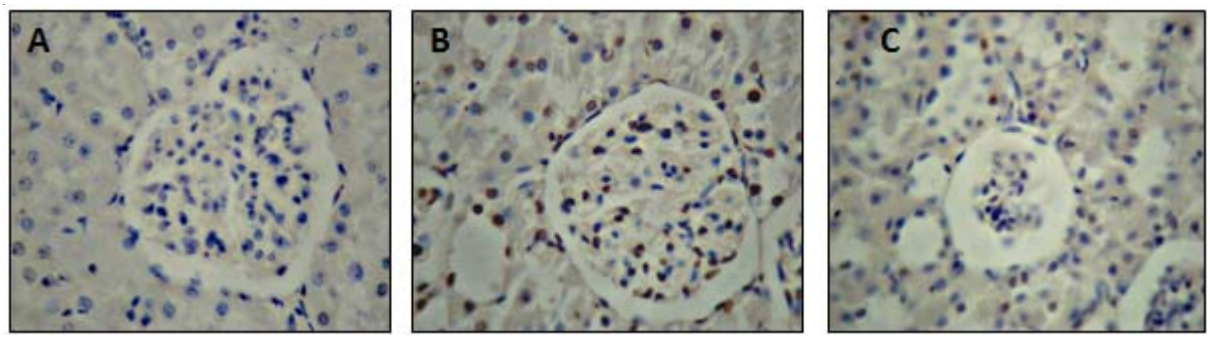

Figure 4. Tunel staining of renal tissues by In Situ Cell Death Detection Kit. **A:** control rats, **B:** diabetic nephropathy rats, **C:** diabetic nephropathy rats that were administrated GSPE. Magnification × 400

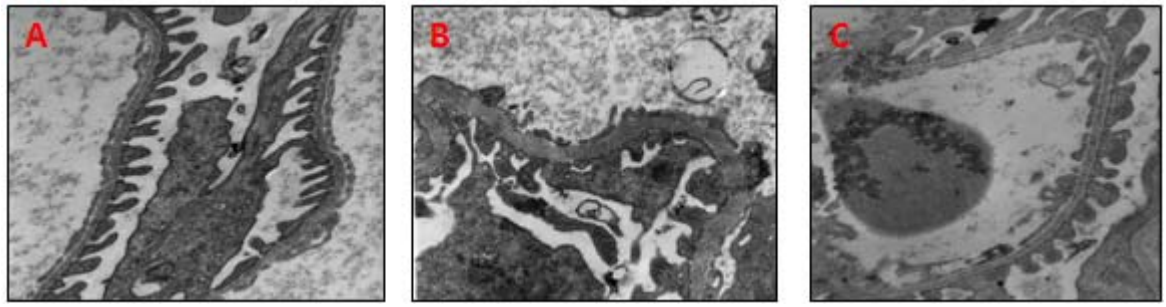

Figure 5. Transmission electron micrographs of representative glomerular basement membrane and podocyte. **A:** control rats, **B:** diabetic nephropathy rats, **C:** diabetic nephropathy rats that were administrated GSPE. Magnification ×21000

Effects of GSPE on Renal lipid peroxidation content

The renal lipid peroxidation content in diabetic nephropathy rats is statistically significantly higher in comparison to control rats **(Figure 6)**. GSPE administration results in a significantly lower renal lipid peroxidase content compared to the untreated diabetic nephropathy rats.

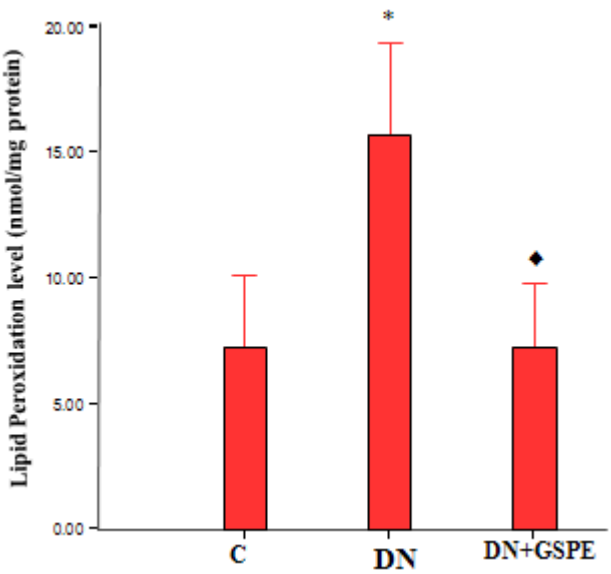

Figure 6. Renal level of lipid peroxidation. $^{*}p$ <0.05 as compared with control; $^{f\&}p$ <0.05 as compared with diabetic nephropathy.

Discussion

In diabetes mellitus, alternating hyperglycemia is an important cause for the development and progress of diabetic complications, such as diabetic nephropathy, since hyperglycemia intensifies oxidative stress correlated to reactive oxygen species (ROS), which contributes to cellular cellular dysfunction and induces apoptosis [8].

In this study, we observed a significant increase in the kidney weight in the diabetic nephropathy rats. The kidney excessive growth is assigned to specific factors such as glucose over-utilization and consecutive boost in rise uptake, glycogen accumulation, lipogenesis, and protein synthesis in the kidney tissue [16, 17]. However, increased mesangial and tubular cell hypertrophy might also be a cause for kidney enlargement [18]. The GSPE administered to the diabetic nephropathy group rats successfully prevented the enlargement of the kidney. This result is in agreement with the study of Li et al [19].

In our study were found, an increased 24-hour albumin excretion in diabetic the write of the nephropathy group. Renal glomeruli filtration barrier impairment can cause a rise in albumin excretion in urine. However, the process of this possible mechanism has not been entirely acknowledged. Disturbed basement membrane metabolism suggested among others by the aggressiveness of the pro-oxidative stress in diabetes, may be one of the possible mechanisms. It has been proposed that both, advanced glycosylation end-products (AGE) interchange with AEG receptor and oxidative stress may cause an increase in permeability of vessels in diabetes [20, 21]. In addition, based on the results of Miner [22], proteinuria in diabetic rats is associated with molecular and ultra-structural variations in podocytes and slit diaphragm.

In this study, the level of albumin excretion in GSPE- treated diabetic nephropathy group is significantly lower than that of the untreated diabetic nephropathy rats, but still statistically higher than controls. Our results are similar to those reported in previous studies [12, 23].

In present study, we observed accumulation mesangial matrix in diabetic nephropathy rats. A rise in the mesangial matrix in DN may develop because of an increase in the level of proteins that are often available in these structures and/or proteins collections not normally exist. It has been proven that some mesangial proteins, such as collagen I and III, are expressed only in the final stages of glomerulosclerosis. Other proteins, like fibronectin, are available in the normal mesangium but increase in the expanding mesangium [24]. In addition, many mechanisms, such as cellular hemodynamic and excess production of advanced glycation end products caused by hyperglycemia are responsible for an increase in mesangial matrix [25, 26]. GSPE attenuates this accumulation of mesangial matrix. Our observations are similar to the previous study [23].

Our findings indicated that apoptotic cells were present in glomerular and tubular cells of diabetic nephropathy group rats. Hyperglycemia can generate cellular damage and activate apoptosis in renal cells in vitro [8, 27, 28]. High ambient glucose can create DNA fragmentation [29] and encourages apoptosis-regulatory genes formulation [30]. Diabetic nephropathy could be managed using antioxidant therapy whereby antioxidant act by suppressing apoptosis [31]. The GSPE administration suppressed apoptosis in diabetic nephropathy rats when compared with untreated diabetic nephropathy rats. This result is similar to results of the previous study [32].

In fact, many studies have indicated that essential exchange in the biochemical and biophysical properties of the glomerular basement membrane (GMB) appear in long-term diabetes and consequently result a loss in the glomerular permselectivity. These alternations, in one hand, are associated with the thickening of the GBM, which represents the hallmark of the diabetic glomerulopathy [33, 34], and on the other hand, is the result of improved synthesis and reduced inversion of GBM structural components [35]. Although prodocytes inhibit a valuable part in conserving a normal glomerular permselectivity but a damaged podocyte may direct an abnormal glomerular permeability as well as a structural alternations of GBM integrity [36]. The recent evidence shows that in early diabetes, the podocyte experience damage, dies, separates from the GBM, and departs from the areas of the GBM denuded. The podocytes that are highly expanded extend their foot processes and try to cover the space [37]. In this study, electron microscopic findings indicated that GBM thickness increased in diabetic nephropathy rats. In addition, an irregularity in podocyte foot process dimensions and broadening of foot process was observed. These changes were prevented by treatment with GSPE. These results are in agreement with the previous study [23].

We also observed an increase in the level lipid peroxidation in diabetic nephropathy rats. Based on the results of Ha and Kim studies [20], raised glucose concentration triggers the system of PKC, which consequently creates an increase in lipid peroxidation in diabetes. It is recommended that PKC stimulation induce oxidative stress in renal glomeruli in diabetes. In contrast, other mechanisms such as glucose auto-oxidation, polyol pathway stimulation, and glycation play a vital role only in later periods of peroxidative damage of renal glomeruli [20]. In addition, an excess generation of various radical species may cause an increase of lipid peroxidation. These radicals are known to activate degeneration of DNA, lipids, and carbohydrates leading to hyperglycemia and correspondent glucose auto-oxidation [38]. These results are in agreement with previous finding [39]. In the present study, GSPE significantly decreased lipid peroxidation in the diabetic nephropathy rats. This result was also observed by other authors [12, 40].

Conclusion

Our data suggest that diabetic state caused functional, pathological, and structural changes in kidney. The present study also showed that GSPE efficiently suppressed some of these changes even if its administration was started after the appearance of significant albuminuria. Our findings also support the potential usefulness of antioxidants in the treatment of diabetic nephropathy. It seems that treatment with GSPE prevented the progression of renal injury in diabetic rats.

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