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Full Length Research Paper

Grape seed proanthocyanidin extract ameliorates diabetic nephropathy in rats

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This study examined the effects of grape seed proanthocyanidin extract (GSPE) on kidney of diabetic nephropathy rats. Male Sprague-Dawley rats were divided into three Groups: control, diabetic nephropathy, diabetic nephropathy and received GSPE 500 mg/kg for 6 weeks. At first, 24-h urinary albumin excretion (UAE) was studied two weeks after induction of diabetes then each week until the end of the experimental period in all of groups. At the end, the experimental animals were sacrificed and the right kidneys were collected and were prepared for light microscopy and electron microscopy evaluation. The results showed that the UAE and kidney weight in diabetic nephropathy rats were significantly higher than in control. Also an increase of the mesangial matrix, apoptotic cells, Bax expression, glomerular basement membrane thickness and broadening of foot process in diabetic nephropathy group rats were observed when compared to control group. These features were found to be reversed when the GSPE was administered to the experimental animals. Our results suggest that GSPE can prevent the progression of nephropathy in diabetic rats.

Key words: Apoptosis, diabetic nephropathy, Bax protein, glomerular basement membrane, grape seed proanthocyanidin extract (GSPE).

INTRODUCTION

Diabetic nephropathy (DN) is a leading cause of end stage renal disease and is associated with a significant social and financial burden (Gordois et al., 2004; Wild et al., 2004; Cowie et al., 2006). It is characterized functionally by proteinuria and albuminuria and pathologically by glomerular hypertrophy, mesangial expansion and tubulointerstitial fibrosis, these findings are closely related to the loss of renal function. Accumulating research suggests that oxidative stress plays a key role in the pathogenesis of diabetic nephropathy. In addition, antioxidant administration has been reported to have potentially beneficial effects in the human kidney and experimental diabetes (Horie et al., 1997; Bursell et al., 1999). Oxidative stress has been suggested to play role as a common mediator in apoptosis (Buttke and Sandstrom, 1994; Jee et al., 2005) and in particular diabetic nephropathy, a state in which oxidative stress increased (Horie et al., 1997). Recent reports provide evidence that high ambient glucose can promote apoptosis *in vitro*, suggesting potential cellular damage as a result of hyperglycemia in diabetes *in vitro* (Allen et al., 2003; Kawano et al., 1992).

Grape seed proanthocyanidin extracts (GSPE) derived from grape seeds, have been reported to possess a variety of potent properties including anti-oxidant, anti-inflammation, radical-scavengering and renal protecting activity, anti-tumor and so on (Shao et al., 2003; Vayalil et al., 2004; Houde et al., 2006). It was reported that GSPE had an effect in protecting the kidneys of diabetic rats (Liu et al., 2006). Therefore we investigated the

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occurrence of apoptosis and its mechanism in the diabetic nephropathy and evaluated the effects of the GSPE on apoptosis and pathologic changes in the diabetic nephropathy rats.

MATERIALS AND METHODS

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

Chemicals

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 h prior to the induction of diabetes. Diabetes was induced by a single intraperitoneal injection STZ (50 mg/kg body weight) freshly dissolved in citrate buffer (0.1 M 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 h 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

A total of 30 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, treated diabetic nephropathy group (GSPE, 500 mg/kg body weight). The GSPE was given in normal saline solution by oral gavages for 6 weeks from the beginning of the 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 the fourth week. Rats were kept individually in metabolic cage with access to drinking water for measurement of twenty-four-hour urinary albumin after two weeks 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 (Rasanayagam et al., 1973).

At the end of the experimental, six rats selected randomly from each group then were sacrificed under ether anesthesia. The right kidneys were collected and perfused so as to be free of blood with PBS and were fixed for light microscopy electron microscopy evaluation separately.

Light microscopy

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 µm thickness (an interval of 100 µm) per animal were stained with periodic acid-Schiff (PAS) techniques. Appropriate fields of view from the renal cortex were evaluated for extracllular matrix deposition.

TUNEL staining

Intranucleosomal DNA fragmentation was labeled *in situ* using an In Situ Cell Death Detection Kit (Roche, Germany). Paraffin-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 tamplate-independent manner (TUNEL-reaction). Sections were then reacted with anti-fluorescein antibody conjugated with horse-radish 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.

Immunohistochemistry

Immunohistochemical detection of Bax expression with mouse monoclonal anti- Bax and ABC staining system was performed as described in data sheet. Briefly, Paraffin-embedded tissue sections, 5 µm thick, were used. Slides were deparaffined with xylene and dehydrated in graded concentrations of ethanol. Endogenous peroxidase was quenched with 3% H_2O_2: methanol (1:1) for 30 min at room temperature. Sections were rinsed twice in PBS and then blocked with 1.5% blocking serum in PBS for one hour at room temperature. Primary antibody was: mouse monoclonal anti- Bax (1:100; Santa Cruz Biotechnology). They were diluted in 1.5% blocking serum in PBS and left overnight at 4°C. Sections were washed twice for five minutes in PBS, followed by the addition of biotinylated secondary antibody (Santa Cruz Biotechnology) at a dilution 1/200 in 1.5% blocking serum in PBS for 30 min at room temperature. After washing twice for five minutes in PBS, sections were incubated with AB enzyme for 30 min. After washing, the antibody location was determined with the addition of DAB chromogen. Color development was stopped by washing in water. Sections were counterstained with hematoxylin, dehydrated and mounted in Canada balsam (DPX, Poole, UK) then evaluated by light microscope and brown areas were judged as positive.

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.

Statistical analysis

Data are expressed as the mean ± SE. Statistical significance of differences was assessed with one-way ANOVA by SPSS for Windows (version 18) followed by Tukey´s t-test. $P<0.05$ was

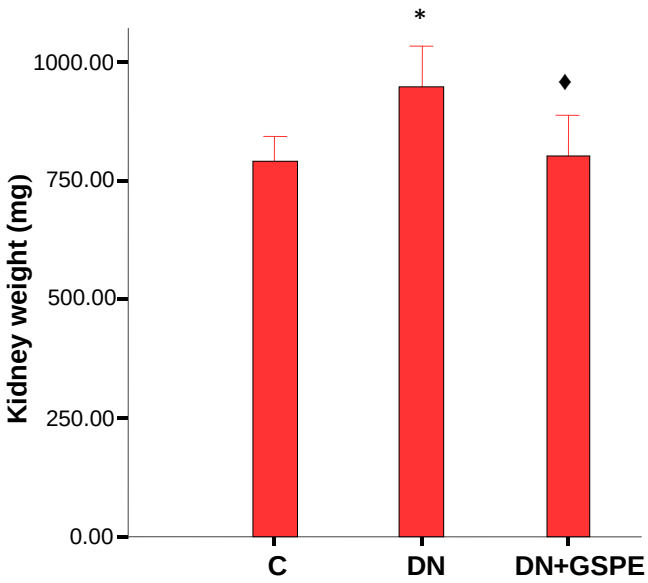

Figure 1. Kidney weight changes between groups, control (C), diabetic nephropathy (DN), diabetic nephropathy treated with GSPE (DN+GSPE) *P<0.05 as compared with control; ♦P< 0.05 as compared with diabetic nephropathy.

assumed as statistically significant.

RESULTS

Effects of GSPE on kidney weight

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

Effects of GSPE on 24 h albumin excretion in urine

A significant increase of 24 h albumin excretion in urine of the diabetic nephropathy group was observed in comparison to the control group. In the diabetic nephropathy group which received GSPE a decreased 24 h albumin excretion in urine was observed in comparison to untreated diabetic nephropathy group; these differences were statistically significant (Figure 2).

Effects of GSPE on renal histology of diabetic nephropathy rats

Light microscopy demonstrated a moderate increase of the mesangial matrix in the glomeruli of most diabetic nephropathy rats in comparison to the control group by an increase in PAS-positive mesangial matrix area (Figure 3). In addition, in diabetic nephropathy rats, focal, segmental hyalinosis and sclerosis were observed. Therapy- with GSPE for 6 weeks reversed the mesangial matrix accumulation that had been established 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

Study with light microscope demonstrated that apoptosis occurred in the tubular epithelial cells and glomerular cells in diabetic nephropathy group. But we did not observe apoptotic cells in tubular and glomerular cells in control group. The apoptotic cells decreased in GSPE-treated diabetic nephropathy group when compared to the untreated diabetic nephropathy rats (Figure 4).

Effects of GSPE on Bax expression in diabetic nephropathy

Immunohistochemical study using anti-Bax antibody showed intensified staining in glomerular and tubular epithelial cells in untreated diabetic nephropathy rats. In contrast glomerular and tubular epithelial cells were not found in control rats. GSPE administration decreased Bax expression in glomerular and tubular cells of diabetic nephropathy rats (Figure 5).

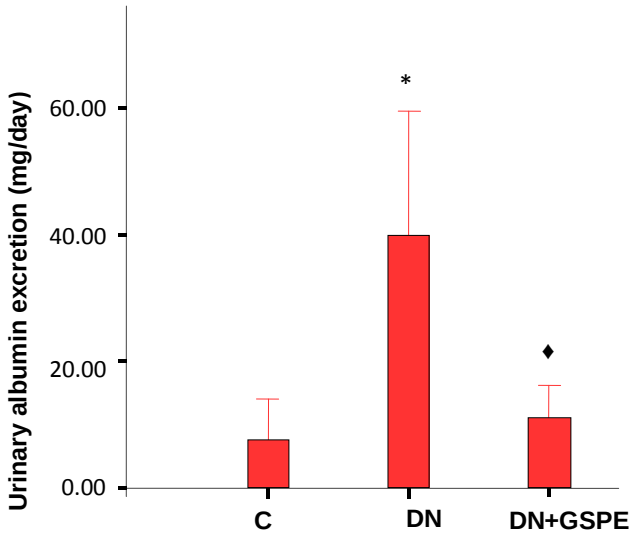

Figure 2. Changes 24 h albumin excretion (UAE) during the experimental period between different groups; *P<0.05 as compared with control; ♦P< 0.05 as compared with diabetic nephropathy.

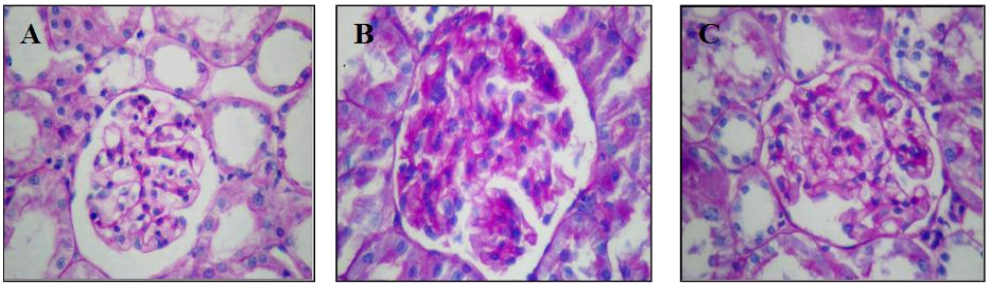

Figure 3. Light microscopy of renal tissue. PAS staining of glomeruli from (A) a control rat, (B) a diabetic nephropathy rat and (C) a diabetic nephropathy rat that was administrated GSPE (×400).

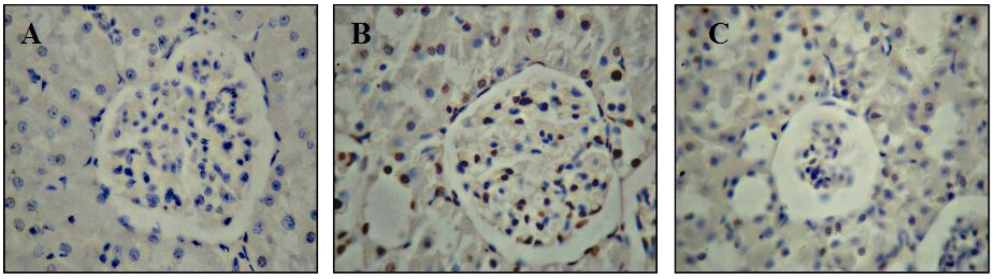

Figure 4. TUNEL staining of renal tissues by *In situ* cell death detection kit. (A) Control rats, (B) diabetic nephropathy rats, (C) diabetic nephropathy rats that were administrated GSPE (×400).

Effects of GSPE on ultrastructure of glomeruli in diabetic nephropathy

Control rats showed a thin glomerular basement membrane (GBM) with relatively spread filtration slit pores. In contrast, in diabetic nephropathy rats we observed a thicker GBM than in control group rats. In addition, we observed irregularity in podocyte foot process dimensions and broadening of foot process.

These effects were attenuated by treatment with GSPE

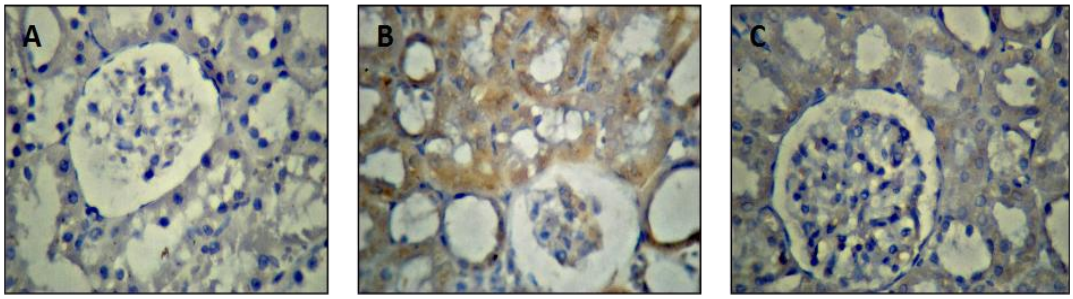

Figure 5. Immunohistochemical staining of bax. Marked immunostaining was observed in glomeruli of (A) control rats, (B) diabetic nephropathy rats, (C) diabetic nephropathy rats that were administrated GSPE (×400).

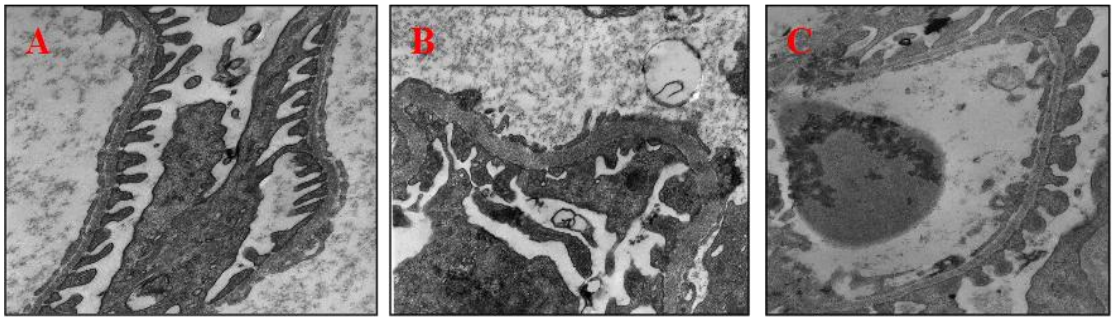

Figure 6. Transmission electron micrographs of representative glomerular capillary loops. (A) Control rats, (B) diabetic nephropathy rats, (C) diabetic nephropathy rats that were administrated GSPE (×21000).

(Figure 6).

DISCUSSION

In diabetes mellitus, uncontrolled hyperglycemia is a high risk factor for diabetic complication development and progression, including diabetic nephropathy, because hyperglycemia increases oxidative stress such as ROS, which leads to cellular dysfunction and induces apoptosis (Allen et al., 2003).

In this study we observed a significant increase in the kidney weight in the diabetic nephropathy animals. The kidney enlargement is attributed to certain factors like glucose over-utilization and subsequent enhancement in increase uptake, glycogen accumulation, lipogenesis and protein synthesis in the kidney tissue (Sun et al., 2002; Peterson et al., 1971).

Also it might be due to increased mesangial and tubular cell hypertrophy (Bulut et al., 2001). 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. (2008).

In our study were found, an increased 24 h albumin excretion in diabetic nephropathic group urine. Increased albumin excretion in urine is caused by renal glomeruli filtration barrier damage, but the mechanism of this phenomenon has not been thoroughly recognized. Disturbed basement membrane metabolism evoked among others by the severity of pro-oxidative stress in diabetes may be one of the possible mechanisms. It has been stated that AGE interaction with AEG receptor and oxidative stress may affect increased permeability of vessels in diabetes (Ha and Kim, 1995; Ceriello et al., 1993). Also, according to results of Miner (2003), proteinuria in diabetic rats is related to molecular and ultrastructural changes in podocytes and slit diaphragm. In this study, the level of albumin excretion in GSPE-treated diabetic nephropathy group is significantly lower than the untreated diabetic nephropathy rats, but still statistically higher than the control group. Our results are similar to those reported in previous studies (Li et al., 2009; Liu et al., 2006).

In the present study we observed accumulation mesangial matrix in diabetic nephropathy rats. Increases in the mesangial matrix in DN may arise due to increases in the levels of proteins that are normally present in these structures and/or accumulation proteins not normally present. It is evident that some mesangial proteins, such as collagen I and III, are expressed only in the late phases of glomerulosclerosis. Other proteins, such as fibronectin, are present in the normal mesangium but

increase in the expanding mesangium (Zeisbert et al., 2002). In addition to in diabetes, many mechanisms, such as cellular hemodynamic, and increase of formation of advanced glycation end products (Nishikawa et al., 2000) caused by hyperglycemia account for mesangial increase (Macedo et al., 2002). GSPE attenuates this accumulation mesangial matrix. Our observations are similar to a previous study by Li et al. (2009).

Our findings indicated apoptotic cells present in glomerular and tubular cells of diabetic nephropathy group rats. Hyperglycemia can trigger apoptosis in renal cells *in vitro* (Allen et al., 2003; Verzola et al., 2002). High ambient glucose can induce DNA fragmentation (Ishii et al., 1996) and stimulate expression of apoptosis-regulatory genes (Ortiz et al., 1997). Antioxidant therapy may be beneficial in preventing the development of diabetic nephropathy. Antioxidants might inhibit the development of diabetic nephropathy by suppressing apoptosis (Lee et al., 2007). Apoptotic cells were decreased in GSPE-treated diabetic nephropathy rats when compared with untreated diabetic nephropathy rats in this study.

High glucose levels cause ROS-dependent apoptosis of mesangial cells and tubular epithelial cells via Bax-mediated mitochondrial permeability and subsequent cytochrome c release (Kang et al., 2003; Verzola et al., 2002). We observed an increased Bax expression in glomerular and tubular cells diabetic nephropathy rats in comparison to control group rats. GEPE decreased Bax epression in diabetic nephropathy group rats. Our findings are similar to a previous study by Piro et al. (2002).

As a matter of fact, many studies have established that important alterations in the biochemical and biophysical properties of the GBM occur in long-term diabetes and follow, or are concomitant with, the loss of the glomerular permselectivity. These alterations correspond mostly to the thickening of the GBM which constitutes the hallmark of diabetic glomerulopathy (Osterby, 1986; Schleicher and Olgemö11er, 1992) and is the result of enhanced synthesis and decreased turnover of GBM structural components (Fogo, 1999). Also podocytes play an important role in the maintenance of normal glomerular permselectivity. Podocyte injury may lead to abnormal glomerular permeability and to structural alterations of GBM integrity (Stackhouse et al., 1990). Recent evidence shows that in early diabetes, the podocyte suffers injury, dies, separates from the GBM and leaves areas of the GBM denuded or the podocytes, that are over-stretched, broaden their foot processes and try to cover the space (Pagtalunan et al., 1997). In this study electron microscopic findings indicated that GBM thickness increased in diabetic nephropathy rats; also 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 a previous study by Li et al. (2009).

Conclusions

Our data suggest that diabetic state caused changes functional, pathological and structural in kidney and also the present 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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