Research Article

ELLAGIC ACID AMELIORATES THE PROGRESSION OF NEPHROPATHY IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

Diabetic nephropathy is one of the major micro vascular diabetic complication which results in the end stage renal disease requiring kidney dialysis or transplantation. The current study was designed to evaluate the nephro protective effect of ellagic acid in streptozotocin (STZ) induced diabetic rats. Diabetes was induced by administration of streptozotocin (55mg/kg, i.p) to overnight fasted male Wistar rats. Ellagic acid (50 mg/kg and 100 mg/kg) was administered orally by dissolving in 0.2% dimethyl sulfoxide every day for a period of 4 weeks. *Invitro* inhibition of advance glycation end products (AGE's) was evaluated. Histopathology of kidney was done by hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) staining. Diabetic rats showed significant elevation of fasting blood glucose, BUN, creatinine and significant decrease in the levels of albumin and total protein levels when compared to normal control rats. Moreover, renal tissue SOD, GSH and catalase were significantly decreased with increase in lipid peroxidation levels. Ellagic acid treatment significantly ($P<0.01$) counteracted all the altered biological parameters. Histopathological findings of kidney further supported the protective effect of ellagic acid administration. Ellagic acid shows renal protection in diabetic rats by its antihyperglycaemic, antioxidant, reducing nitro-oxidative stress and AGE inhibitory activity and thus can be a promising agent for prevention of diabetic nephropathy.

Key words: Ellagic acid, Diabetic nephropathy, Renal protection, Streptozotocin, Advanced glycation end products.

INTRODUCTION

Diabetic nephropathy (DN), is the most common and specific micro vascular complication of diabetes which remains a major cause for end stage renal disease (ESRD)¹. DN is clinically defined as progressive development of renal insufficiency in the setting of chronic of hyperglycemia. The functional disorders in DN are manifested by early microalbuminuria, renal hyperfiltration, hyperperfusion and increasing capillary permeability to macromolecules and proteinuria with or without chronic renal insufficiency that leads to ESRD².

Hyperglycaemia is the key player in the development of DN³ acting through formation and accumulation of advanced glycation end products, activation of protein kinase C, acceleration of the polyol pathway, production of reactive oxygen species and over-expression of transforming growth factor- β . In addition to these factors, the emerging roles of inflammatory processes, such as over-expression of cell adhesion molecules and chemokines, which induce leukocyte infiltration, are recognized in DN⁴.

Ellagic acid, a dimeric derivative of gallic acid is found in numerous fruits and vegetables including blackberries, raspberries, strawberries, cranberries, walnuts, peanuts, pomegranates, wolfberry and other plant foods in either its free form, as EA-glycosides or bound as ellagitannins⁵. It is believed to have anti-oxidant, hepatoprotectant, anti-inflammatory, anti-carcinogen and antimutagenic properties⁶. This study was undertaken to explore the effect of ellagic acid in the development of nephropathy in type 1 diabetic rat model.

MATERIALS AND METHODS

Drug solution preparation

Streptozotocin (STZ) purchased from Sigma-Aldrich was dissolved in ice cold 0.1M sodium citrate buffer just prior to use and injected dose of 55mg/kg, ⁷ i.p. Ellagic acid was dissolved in 0.2% w/v dimethyl sulfoxide and administered to rats orally using per oral tube daily. The solutions were freshly prepared every day before dosing the animals.

Experimental design and drug treatment

Healthy male Wistar rats weighing about 175-225g were used in the study. The use of animals in these experiments was authorized by IAEC (Institutional Animal Ethics Committee). All animals were housed in an air-conditioned room at $24\pm1^{\circ}\text{C}$ with a 12-h light/dark cycle and allowed ad libitum access to water and standard pelleted diet.

48h after STZ administration rats with fasting serum glucose more than 250mg/dl were selected. Rats were divided into four groups Group I – normal control; Group II – diabetic control; Group III and IV diabetic rats treated with ellagic acid 50 & 100mg/kg, p.o.⁸

At the end of study, individual rats were placed in metabolic cages for 24h urine collection and blood was collected from retro-orbital sinus of overnight fasted animals and was immediately centrifuged for separation of serum and stored for analysis. Rats were sacrificed by over dose of ether and kidneys were removed and weighed, the ratio of weight of kidney to body was calculated and termed as kidney index. One kidney

was fixed in 10% (w/v) neutral buffered formalin solution for histopathological examination and another was frozen to -20°C for preparation of tissue homogenate.

Biochemical parameters

Parameters were measured by using commercially available kit (Autospan, Gujarat, India), glucose by glucose oxidase method⁹, albumin by bromo cresol green dye method¹⁰, total proteins by modified biuret method¹¹, creatinine by Jaffe's reaction¹², BUN by berthelot method¹³. Same principle and reaction was used for urine estimation of creatinine and albumin as per the instructions given in the kit. Nitric oxide was estimate by nitrite assay¹⁴. UAER was calculated by formula¹⁵: $\text{UAER (mg/24 h)} = \frac{24 \text{ h total volume of urine (L)} \times \text{urinary albumin levels (mg/L)}}{\text{Clearance of creatinine (CCr)}}$ ¹⁶ was calculated by formula: $\text{CCr (ml/min)} = \frac{[(\text{Urine Cr in mg/dl}) \times \text{Urine volume (ml)}]}{[(\text{Serum Cr in mg/dl}) \times 1440 (\text{min})]}$.

Tissue estimation

Isolated kidneys were homogenized in ice cold 20 mM Tris-HCl buffer (pH 7.4) and the homogenates were then centrifuged at 10,000 g for 10 min at 4°C . The supernatants were collected and used for assessment of Lipid peroxidation (as malondialdehyde) levels measured by the method of Dilek Y¹⁷, antioxidant defense system assays superoxide dismutase (SOD) were determined according to the method of Marklund and Marklund¹⁸, catalase (CAT) by the method of Aebi¹⁹, reduced glutathione (GSH) by the method of Jollow²⁰.

Histopathology

Kidneys fixed in formalin solution were embedded in paraffin sectioned and stained with haematoxylin – eosin and periodic acid-schiff stain (PAS) to examine the pathological changes occurred in glomeruli and tubules using light microscopy under 400x.

In-vitro assay of AGE

Assay was carried out by the method of Brownlee²¹

Statistical analysis

All data were expressed as mean $\pm$ SEM and analyzed with one way analysis of variance between the groups and followed by Dunett's Multiple comparison test to assess differences between the groups and region means. $P < 0.05$ were considered as significant.

RESULTS

Serum biochemical parameters and kidney index

At the end of 4week study DC group showed significant increase in fasting serum glucose, creatinine, blood urea nitrogen and nitric oxide and significant decrease in serum albumin and total proteins compared to NC. Ellagic acid treatment significantly decreased the elevated fasting serum glucose, creatinine, blood urea nitrogen and nitric oxide and increased serum albumin and total proteins compared to the DC group (Figure 1). Similarly treated group showed significant decrease in ratio of body weight to kidney weight comparable with the diabetic control group (Table 1).

A

B

C

D

Figure 1: Effect of oral administration of ellagic acid on %change in glucose(A), albumin(B), total proteins (C), creatinine(D), BUN (E), nitrite/nitrate (F) levels in STZ induced diabetic rats

Values are expressed in mean $\pm$ SEM, n=6. *(p<0.05), ** (p<0.01), *** (p<0.001) when compared with diabetic control group by using one way ANOVA followed by Dunnett's multiple comparison test

Urine parameters

At the end of 4-week study urine albumin and urinary albumin excretion rate were significantly higher in DC compared to NC. Along with that urinary creatinine and creatinine clearance were

decreased in DC compared with NC. Treatment with ellagic acid showed significant reduced urine albumin and urinary albumin excretion rate and significant increase in urinary creatinine and creatinine clearance compared to DC (Figure 2, Table 1)

Figure 2: Effect of oral administration of ellagic acid on urinary albumin (A) and creatinine (B) levels in STZ induced diabetic rats

Values are expressed in mean $\pm$ SEM, n=6. *(p<0.05), ** (p<0.01), *** (p<0.001) when compared with diabetic control group by using one way ANOVA followed by Dunnett's multiple comparison test

Table 1: Effect of oral administration of ellagic acid on kidney index creatinine clearance, urinary albumin excretion rate levels in STZ induced diabetic rats

Sl.no	Groups	Kidney index (mg/g)	Ccr (ml/min)	UAER (μ g /24h)
1.	Normal control	4.24 $\pm$ 0.17**	0.24 $\pm$ 0.017***	0.04 $\pm$ 0.002***
2.	Diabetic control	7.52 $\pm$ 0.13	0.14 $\pm$ 0.008	1.35 $\pm$ 0.041
3.	Ellagic acid 50mg/kg	4.29 $\pm$ 0.11**	0.22 $\pm$ 0.018**	1.03 $\pm$ 0.038**
4.	Ellagic acid 100mg/kg	4.35 $\pm$ 1.09**	0.21 $\pm$ 0.017**	1.08 $\pm$ 0.079**

Values are expressed in mean $\pm$ SEM, n=6. *(p<0.05), ** (p<0.01), *** (p<0.001) when compared with diabetic control group by using one way ANOVA followed by Dunnett's multiple comparison test.

Kidney tissue estimation

At the end of 4-week study significant increase in MDA and decrease in SOD, CAT, GSH was found in DC compared to the NC. Treatment with ellagic acid significantly decreased MDA and increased in SOD, CAT, GSH compared to the DC.

Figure 3: Effect of oral administration of ellagic acid on renal SOD (A), CAT (B), GSH (C) and MDA (D) levels in STZ induced diabetic rats. Values are expressed in mean $\pm$ SEM, n=6. * (p<0.05), ** (p<0.01), *** (p<0.001) when compared with diabetic control group by using one way ANOVA followed by Dunnett's multiple comparison test.

Figure 4: Histological analysis of rat kidneys in NC (1A), DC (1B), EA50 (1C), EA100 (1D) groups by H & E staining and histological analysis of rat kidneys in NC (2A), DC (2B), EA50 (2C), EA100 (2D) groups by periodic acid Schiff (PAS) staining $\times 400$.

Histopathological studies

PAS and H& E stained kidney specimens showed normal glomerulus in NC group (Figure 4 1A, 2A). The kidney of the diabetic rat developed pathological changes in the glomerulus such as mesangial cell expansion (Figure 4 1B, indicated in arrow), mesangial cell expansion with diffuse mesangial sclerosis (Figure 4 2B, indicated in arrow) EA50 and EA100 groups showed minimal morphological changes in decreased mesangial matrix accumulation and mesangium appeared normal (Figure 4 1C and D, 2C and D).

In-vitro assay of AGE

Fluorescence intensity of glycated end products was significantly higher after incubation with glucose. Ellagic acid (10–100 μ g/ml) inhibited the glycation of bovine serum albumin and subsequent formation of fluorescent glycated products in a concentration dependent manner. Aminoguanidine was used as a positive control. IC₅₀ value of ellagic acid and aminoguanidine for inhibition of advanced glycated end products was found to be 55 μ g/ml and 50 μ g/ml respectively.

Figure 5: *In vitro* estimation of inhibition of advanced glycated end products

DISCUSSION

Induction of diabetes using STZ 55mg/kg, i.p resulted in hyperglycemia that was accompanied by loss of body weight, increase in kidney weight to body weight ratio and progressive renal damage as indicated by polyuria, elevation in serum creatinine level, increase in urinary albumin excretion, proteinuria and reduction in creatinine clearance as indicator of GFR like the studies reported by *Trachtman H*²²

In the present study administration of ellagic acid for 4 weeks to rats 48hours post induction of diabetes showed amelioration of the damage of kidneys as assessed by reduced serum creatinine, BUN, urinary total protein, urinary albumin. Increased serum albumin and serum total proteins were also seen. EA also showed significant decrease in hyperglycemia. Mechanism of action may be through potentiation of pancreatic secretion of insulin from beta cells of islets or due to enhanced transport of blood glucose to the peripheral tissue as reported in previous study⁸

One of the major consequences of hyperglycemia is the formation of reactive oxygen species apart from glycation, AGE accumulation, polyol pathway and cytokines with hyperglycemia.

Reactive oxygen species initiate the inflammatory cascade with subsequent activation of nuclear factor- κ B (NF- κ B). This transcription factor NF- κ B may stimulate the expression of numerous genes and inflammatory mediators including inducible NOS, ICAM-1 and numerous cytokines as TNF- α and interleukin-1 β . This can result in nephropathic changes.

In the current study concentrations of enzymes responsible for antioxidative defense mechanisms such as catalase, glutathione and superoxide dismutase, were found to be lower in kidney homogenate of diabetic rats with increase in lipid peroxidation levels. Along with that increase in serum NO and AGE was also seen which are in harmony to those observed by other investigator^{23, 24}.

Study reports²⁵ have acknowledged the antioxidant property of ellagic acid by inhibiting the generation of superoxide and hydroxyl free radicals in both enzymatic and non-enzymatic

systems by means of its metal-chelating property, thus providing protection against lipid peroxidation as in our study showed treatment with ellagic acid elevated the SOD, GSH and CAT enzyme level and ameliorated the TBARS.

Ellagic acid administration has shown to reduce elevation NO levels in serum adding to the reduction of nitrosooxidative stress and protecting kidney against damage.

Hyperglycemia promotes the formation of AGE. Interacting with its specific receptor, AGE induces the generation of oxygen species (ROS) and mediates its action via oxidative stress²⁷. In this study ellagic acid has exhibited *in-vitro* inhibition of AGE accumulation there by reducing the oxidative stress with the accumulation of AGE. The reduction in serum advanced glycation end products obtained by ellagic acid treatment may be due to its ability to inhibit the free radicals auto oxidation of glucose which binds to the albumin to form advanced glycation end products. These observations from our study suggest a potential clinical use of ellagic acid in the prevention of diabetic complications by inhibition of advanced glycation end products, nitric oxide and improving the free radical defense system besides its antihyperglycemic effect.

Histopathological evaluation of diabetic nephropathy kidneys showed characteristic changes in renal structure, including renal hypertrophy, increased thickening of GBM (PAS staining), and glomerulosclerosis. Glomerulosclerosis is an eventual pathogenesis of diabetic nephropathy. Similar structural changes have been described in other experimental animal studies and humans²⁷. Thus, confirming the renal damage of STZ at the dose used by the similar previous studies.

Treatment with ellagic acid prevents the pathological changes in the preventive studies and reduced the structural irregularities in the diabetic nephropathic kidneys providing supportive evidence of the nephroprotective activity of ellagic acid.

In conclusion, ellagic acid administration (50mg/kg and 100mg/kg, p.o) showed restoration of the altered biochemical and histopathological parameters in the diabetic rats. Ellagic acid provided renoprotection via maintenance of glucose level, inhibition of advanced glycation end products accumulation, antioxidant action and the suppression of NO. Thus, ellagic acid

has the potential to act as an adjunct drug for the prevention of diabetic nephropathy.

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