Original paper

Potentially beneficial effects of ethyl-pyruvate on diabetic nephropathy: an experimental and ultrastructural study

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Oxidative stress is one of the main reasons of the diabetic nephropathy, which is a complication of the diabetes mellitus (DM). The aim of this study was to investigate the possible role of ethyl-pyruvate (EP) in streptozotocin-induced diabetic rats' kidney.

Four groups (n = 8) male Wistar albino rats were used as follows: controls group rats were received only sodium citrate buffer solution by intraperitoneally (ip). EP group was given 50 mg/kg EP ip. DM group, diabetes formed by inducing streptozotocin. DM + EP group, received 50 mg/kg EP ip. All animals received daily treatment for 14 days, and at the end of the study, the kidneys were removed: the left kidney of the rats for malondialdehyde (MDA) analysis and the right kidney for histological examination.

There was normal appearance of the kidney tissues in the control and the EP administered groups. In DM group, there was evident basement membrane thickening, enlargement of mesangial matrix; swelling in some tubular epithelial cells was noticeable. In DM + EP administered group, nearly control group appearance as well as relatively thickening in the glomerular basal membrane were detected.

The antioxidant effect of ethyl-pyruvate improved the renal structures in DM + EP group.

Key words: ethyl-pyruvate, diabetes mellitus, nephropathy, streptozotocin.

Introduction

Diabetes mellitus (DM) is a widely seen endocrine disorder. It is characterised by hyperglycaemia and susceptible to chronic complications affecting kidney (diabetic nephropathy), vasculature and eyes (diabetic retinopathy) [1]. Diabetic nephropathy is also characterized by renal hypertrophy and extracellular matrix accumulation as well as glomerular sclerosis which gives rise to proteinuria and renal failure. The exact possible etiological factors underlying pathophysiological mechanism of the diabetic nephropathy (DN) is still remain unclear. It has been suggested that hyperglycaemia plays an important role in the pathogenesis of the diabetic nephropathy by means

of producing oxidative stress and advanced glycation end-products (AGEs) [2, 3].

Accumulated evidence has put forward three major pathways implied in the development of the diabetic nephropathy in the last two decades [4]. One of them is formation of the AGEs by hyperglycemia which induces dysfunction of the glomerular cells and activation of macrophages [5, 6]. The receptor of the advanced glycation end-products (RAGE) pathway is virtually involved in the transduction of the subsequent cell signalling related to inflammation and oxidative stress [7]. It was reported that overproduction of free radicals depends on either oxidative stress or secondary to oxidative stress in DM patients and rat model of DM. Free radicals lead to tissue damage by nuclear factor kappa B (NF-κB). It was shown that NF-κB plays a key role in the formation of DN and iNOS activation [8, 9]. Several studies reported that NF-κB implied in pathophysiological mechanism of the DN. In addition, it has been shown that the ethyl pyruvate plays a role in the prevention of activation of NF-κB. It is possible that ethyl pyruvate inhibits activation of NF-κB simply by scavenging H_2O_2. Nevertheless, some observations have put forth that ethyl pyruvate mediated inhibition of NF-κB activation is likely to involve more than one antioxidant effect [10, 11].

Antioxidants are molecules that struggle with oxidative stress and inflammation. Vitamins (C, E), resveratrol, ginseng, green tea and ethyl-pyruvate are some of the important ones of these antioxidants [12, 13]. The potent antioxidant effects of EP have been shown in some studies [14]. EP is basically derived from pyruvic acid [15]. It has also been shown that this molecule improves organ dysfunction in some situations, such as sepsis [16], ileus [17], acute pancreatitis [18] and organ ischemia and hemorrhagic shock [19, 20, 21]. In addition, EP ameliorates the adverse effects of the streptozotocin induced diabetic rats' testes [22] and liver [23]. EP has been determined to be effective as pyruvate. Moreover, it has been suggested to scavenge phenoxy radicals. However, the biological acting of this molecule as a free radical scavenging is still unknown [11].

Still, it is not known if ethyl pyruvate can provide protection against experimental induced DM. Therefore, the present study was carried out to evaluate the role of EP in streptozotocin induced diabetic nephropathy.

Materials and methods

Seven or eight-week old male Wistar albino rats each weighing about 200–240 g, were used in our study. The rats were provided from the experimental Animal Laboratory Medical Research Center of Dicle (DUSAM) and kept in a temperature-controlled, 60% ratio moisture room at 23 ±2°C with a 12-h light/12-h dark cycle with free access to tap water and standard laboratory rat chow. We conducted the study after receiving the approval of Ethics Review Committee for Animal Experimentation, Dicle University, and carefully followed the guidelines "Protection of Animal Rights" during our experimental process established by NIH. Prior to study, body mass and blood glucose levels of rats were measured.

Experimental procedure

Streptozotocin was used to form experimental diabetes in rats. STZ (Sigma, USA) was induced by intraperitoneal application of 45 mg/kg that had previously dissolved in 0.1 M sodium citrate buffer at pH 4.5 [24]. The same volume of a sodium citrate buffer (pH 4.5) solution was induced to non-diabetic rats peertimely by intraperitoneal injection (ip). Seventy two hours after STZ injection blood samples were collected from the tail vein, and blood glucose levels were determined by a glucometer (Medisense Optimum Glucometer Roche Diagnostic, Germany). Rats with blood glucose levels higher than 300 mg/dl were considered to be diabetic and enrolled into the study.

Fourteen weeks after inducing diabetes, diabetic and non-diabetic rats were divided into two groups. The groups were assigned as follows: Group 1: control group (n = 8) rats received only sodium citrate buffer solution by intraperitoneal application; Group 2: ethyl-pyruvate (EP) group (n = 8) received 50 mg/kg EP pi 12 h at a dosing interval for totally 14 days; Group 3: DM group (n = 8) diabetes was formed by inducing STZ pi; Group 4: DM+EP group (n = 8), diabetes was formed by inducing STZ and also received 50 mg/kg EP pi 12 hours at a dosing interval for totally 14 days by intraperitoneal injection. EP dissolved in Ringer's solution (130 mmol/l Na^+, 4.0 mmol/L K^+, 2.7 mmol/l Ca^2, and 109 mmol/l Cl^-, pH 7.0) was administered to rats in Groups 2 and 4, 50 mg/kg 12 h at a dosing interval for totally 14 days by ip.

Peertimely, the same volume of ringer lactate solution was administered to the other group of rats. On the 15th day the rats were sacrified under Ketamin HCl (100 mg/kg, intra muscular [i.m.]) and Xylazine (15 mg/kg, i.m.) anesthesia by opening thorax wall and incision on heart. The kidneys were removed: the left kidneys of the rats for MDA analysis and the right kidneys for histological examinations. The tissues examined under light microscope were photographed (Nikon Eclipse 80i, Japan).

The right kidneys were removed from eight animals in each group. The biopsy samples from the kidneys were fixed in neutral buffered formaline solution. After 24 hours of fixation, the tissues were

dehydrated in decreasing alcohol series, cleared in xylol solution and embedded in paraffin blocks. Four-five μm sections were cut from paraffin embedded blocks and stained with Periodic Acid-Schiff (PAS) for examination of morphologic structure of kidneys. The samples were examined under photomicroscope (Eclipse 80i, Nikon, Japan). For electron microscopic examination, the biopsy samples were fixed in 2.5 % gluteraldehyde solution, then posfixed with osmium tetroxide dehydrated in increasing alcoholic solutions and embedded in epon resin. Ultrathin sections cut by ultramicrotome were stained with lead citrate and examined by electron microscopy (Jeol JEM 1400, Japan).

Analysis of malondialdehyde

The spectrofotometric method which was used before by Buege-Aust was preferred to determine the MDA tissue levels. The MDA results that we obtained were named nmol MDA/gram tissue [25].

Statistical analysis

SPSS (Version 11.0. Chicago, SPSS Inc., USA) programme was used in statistical analysis. The results were formulated averagely as ± standard deviation. Kruskall-Wallis one-way analysis of variance test was used in the analysis of the histopathologic measurements and MDA levels. The Mann-Whitney U test was applied in case of positive significant conclusion to compare the binary groups with each other. The Mann-Whitney U test was used when comparing diabetic and non-diabetic rats for their body mass and plasma glucose levels. The significance level $p < 0.05$ was approved in all statistical analyses.

Results

MDA levels

The MDA results of all groups are shown in Table I. The MDA levels in diabetic group was found significantly higher when compared to control group and EP group ($p \leq 0.001$). MDA levels in diabetic group which had not received EP was determined to be significantly higher than the diabetic group that received EP ($p < 0.01$).

Light microscopic analysis

In the examinations under the light microscopy, there was normal appearance of the kidney tissues in the control group and the EP receiving groups (Fig. 1A, B). In DM, there was evident basement membrane thickening, narrowing in the Bowman's space, enlargement of mesangial matrix and cell proliferation. Swelling in some tubular epithelium cells which showed hydropic changes, beside the pale stained of cytoplasm the accumulation of glycogen in various diameters and size reacted with PAS (+) in the tubular epithelium cells of corticomedullary area were noticeable (Fig. 1C). In DM+EP administered group, it was detected nearly control group appearance despite hydropic degenerations in minimal levels at the tubular epithelial cells as well as relative thickening in the parietal layer of Bowman's capsule and thickening in the basement membranes of tubules (Fig. 1D).

Transmission electron microscopic analysis

In the ultrathin sections of control group, the podocyte nucleus of the kidney filtration membrane, pedicels, filtration splits, fenestrates of capillary endothelium, Bowman's space and erythrocytes in the capillary lumen were observed in normal histological features (Fig. 2A). Additionally, the apical parts of proximal and distal tubule cells, basal lamina and organelles of the cell as well as macula adherences were seen in normal appearance in control group sections (Fig. 2B).

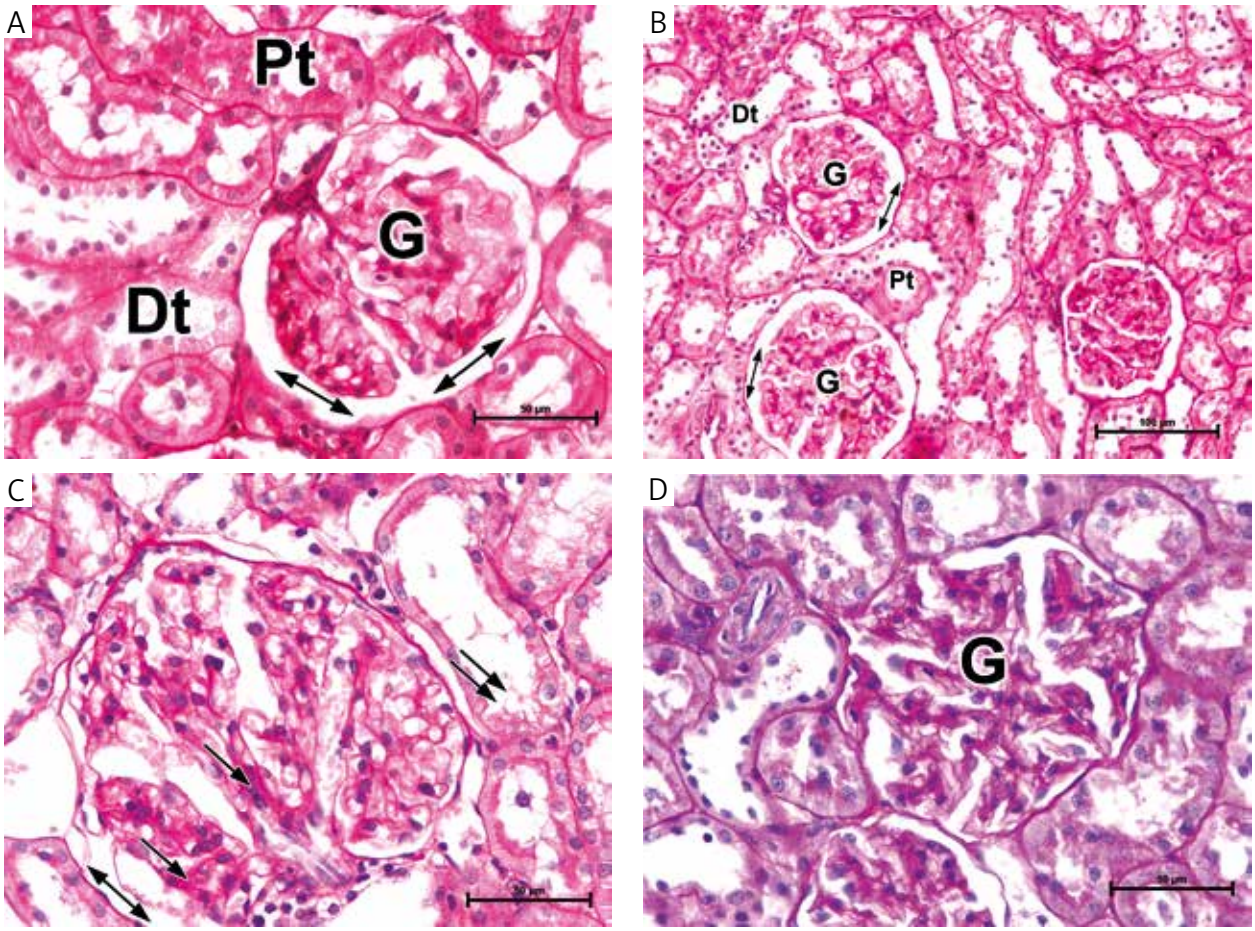
The glomerular structures (Fig. 2C) and proximal and distal tubular structures were detected similar to those in the control group in the ultrathin sections of the EP group (Fig. 2D).

There was obliteration in the slits of the pedicels and capillary fenestrations besides the dilatation in the thin glomerular structure, Golgi cisternas and tubules in DM group sections (Fig. 3A). It was detected that microvilli of proximal tubule cells were lost continuity because of degeneration and also partly enlargements in the tubular basal membrane foldings were seen in another section of DM group. For the conclusion of STZ administration, the regular dispersion of mitochondria in the basal foldings were broken down, and the occurence of autophagic vacuoles were determined (Fig. 3B).

Table I. MDA levels of kidney tissue

Group	MDA* (nmol/gram)
Control	131.9 ±8.2
EP	139.5 ±5.9
DM	225.0 ±23.0a
DM + EP	167.1 ±13.5b

**Kruskal Wallis test $p \leq 0.001$. The results were given as average ± standard deviation*
[a] $p \leq 0.001$ when comparing with control and EP groups
[b] $p < 0.01$ when comparing with DM group
EP – ethyl pyruvate; DM – diabetes mellitus; DM + EP – DM administered ethyl pyruvate
The MDA levels in diabetic group was found significantly higher when compared with control and EP group ($p \leq 0.001$). MDA levels in diabetic group which had not received EP was determined to be significantly higher than the diabetic group received EP ($p < 0.01$).



Dt – distal tubule, Pt – proximal tubule, G – glomerulus, bidirectional arrows – Bowman space, arrows – basement membrane, two arrows – hydropic changes PAS staining

Fig. 1. Panoromic appearance of the glomerular structure in the cortex of kidney of the control (A) and EP groups (B). Basement membrane thickening, hydropic changes and narrowing of the Bowman's space as well as enlargement of the mesangial matrix and cell proliferation were seen in DM group (B). Nearly control group appearance was seen in EP+DM group (D). Bar scale: A, B, C and D is 50 μm, 100 μm, 50 μm and 50 μm, respectively

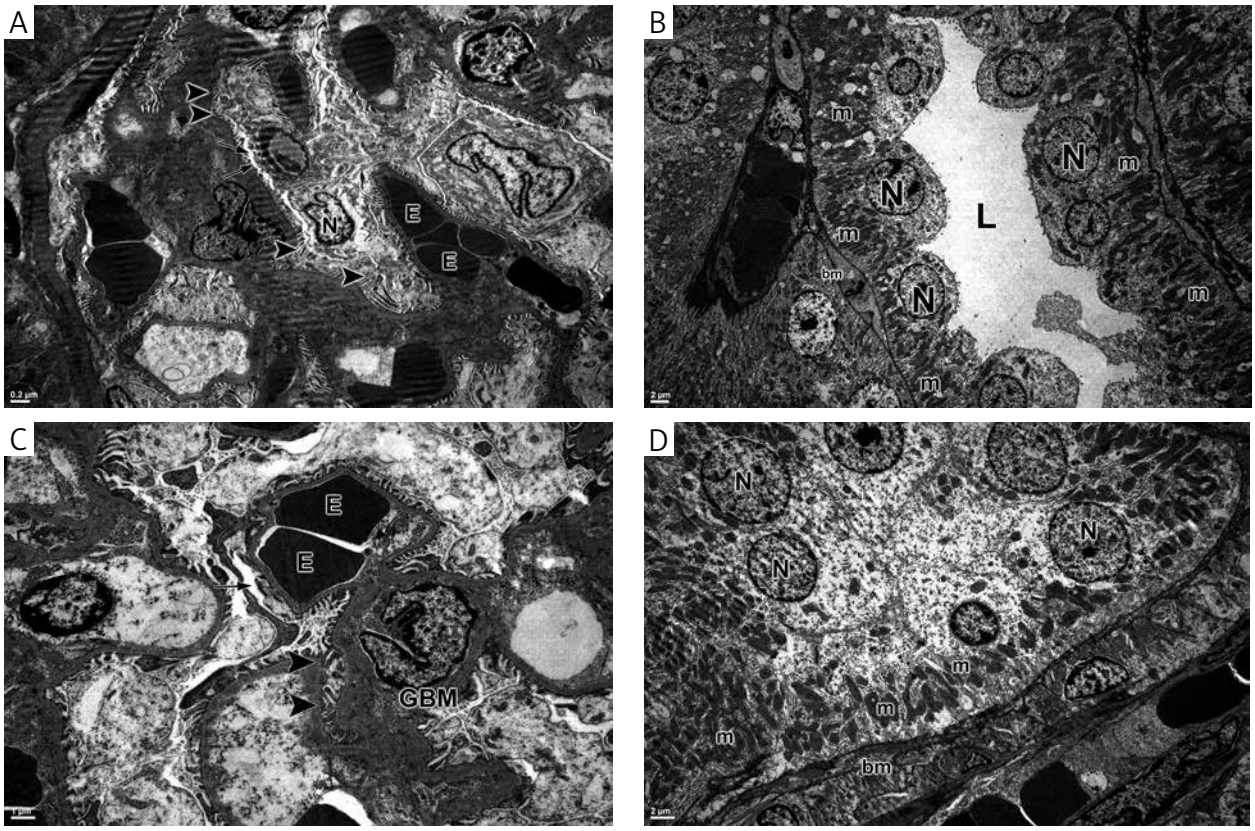
In the group of DM + EP, the ultrastructural appearence of glomerulus, nucleus of the podocytes, pedicels and fenestrated capillaries were nearest to normal; however, the thickening was present partly in some glomerular basement membranes (Fig. 3C). Also in the ultrathin sections of this group, the epithelial cells of the proximal and distal tubules were found similar to those in the control group (Fig. 3D).

Discussion

DM is considered a worldwide public health problem, either in terms of the number of people affected and premature mortality or the costs involved in controlling it and treating complications. It has been reported that DN occurs approximately in 30-40% of diabetes mellitus type 1 (DM-1) [26] and major reason of death in diabetic sufferer. The mechanism of glomerular pathology underlying in DN is still unclear [27]. There is evidence showing that diabetic nephropathy results from metabolic alterations that occur in the renal glomeruli after long exposure to high glycemic levels [28, 29]. In addition to the hemodynamic effects, several non-hemodynamic factors have been identified to have been involved in the pathogenesis of DN [27].

It has been believed that deteriorated peritubular microcirculation and sequential tubular damage are the most supported theories in DN pathogenesis. Raised levels of glucose in ultrafiltrate lead to an increase in glucose absorption of proximal tubules and intracellular glucose deposition. Carboxymethyllysine is advanced glycation end-products as well as consecutive NF-κB elevation is indicated secondary to high levels of intracellular glucose levels. Elevation of nuclear translocation of NF-κB has been shown in DN [12].

However, there have been more studies about the protective role of ethyl pyruvate on other organs: brain, liver, intestine and the studies about experimental diabetic rats with other antioxidants. Otherwise, there would have been very few studies on ethyl



N – nucleus; L – lumen, filtration slits – thick arrows; pedicels and obliteration – arrow head; thin arrows – endothelial fenestrations; bm – basement membrane; E – erythrocyte; mv – microvilli; GBM – glomerular basement membrane

Fig. 2. Panoramic appearance of the glomerular structure (A) as well as the distal tubule cells (B) of the cortex of the kidney of the control group. Normal view of the glomerular structure (C) and distal tubules (D) of the EP group were seen. Bar scale: A, B, C and D is 0.2 μm, 2 μm, 1 μm and 2 μm, respectively

pyruvate with experimental diabetic nephropathy; therefore, so we studied the ethyl pyruvate in the kidney to clarify the protective effects of ethyl pyruvate morphologically.

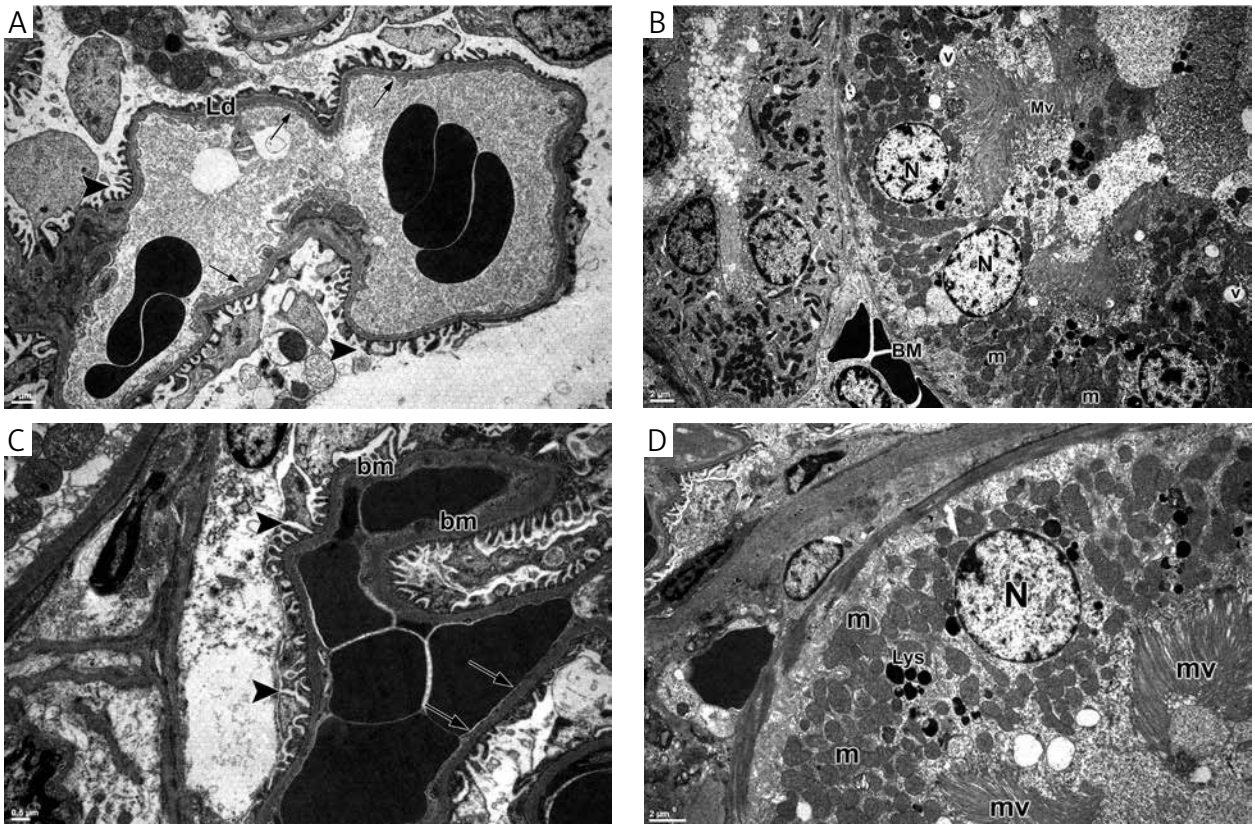
It has been shown that ethyl pyruvate decreases sepsis-induced renal failure. In addition, the inhibition of induction of mRNA level for tissue factor, plasminogen activator inhibitor-1, tumor necrosis factor and tissue plasminogen activator which are believed to have destructive effects in sepsis were also seen [11].

The early changes in diabetic nephropathy are characterised by increased kidney size, glomerular volume and kidney function [30]. However, in the initial stages of diabetic nephropathy, clinical signs and symptoms may be vague or even absent for sometime. In STZ-induced diabetic rats, mesengial enlargement, GBM thickening, proteinuria and, tubular dilatation are observed [12]. Progressive accumulation of extracellular matrix components in both glomerular mesangium and tubulointerstitial structures has been shown in diabetic nephropathy [31].

It has also been observed that glomerular hypertrophy plays a central role in the pathological mechanism of several renal disorders and one of the earliest alterations which are seen during the development of the DN. Glomerular hypertrophy was seen to be exact within 4 days after rats were induced diabetic [26]. Morphometrical analysis of glomerular size has been shown that glomerular size expanded markedly in untreated diabetic rats [32]. Through histological analysis, the expansion of glomeruli has been revelead to be associated with an increase in mesangial matrix and GBM thickness [26].

The protective effect of thymoquinone against renal damage has been demonstrated. In this study, similar protective effects were seen in treatment group in terms of renal functional assesment. Thymoquinone slows down the histopathologic alterations and deterioration. In addition, irbesartan corrected the histopathological alterations more prominently than thymoquinone [27].

In the present study, we determined significant decrease after the treatment of EP, so that the glomerular size, amounts of mesangial matrix and tubular structures were observed nearest to the control group except partly thickening of tubular basement membranes. Therefore, when comparing the antioxidants, EP seems more protective than thymoquinone. Thymoquinone has only reduced but not treated the neg-



N – nucleus; L – lumen, filtration slits – thick arrows; pedicels and obliteration – arrow head; thin arrows – endothelial fenestrations; bm – basement membrane; E – erythrocyte; m – mitochondria; Ld – lamina densa; mv – microvilli; v – autophagic vacuoles; GBM – glomerular basement membrane; Pt – proximal tubule; Lys – lysosome; G – glomerul

Fig. 3. Thickening of the lamina densa layer of the basement membrane is evident in glomerular structure and obliterations in slits between pedicels and capillary fenestrations (A). Additionaly, degenerative changes in the proximal tubule cells with irregular shaped microvilli, and dispersing mitochondria located between the basal infoldings and autophagic vacuoles were seen in DM group (B). The ultrastructural appearance of the glomerulus, pedicels, capillary fenestrations were nearest to the control and EP groups. However, the thickening of the glomerular basement membrane was present in some areas (C) in DM+EP group. In addition, the proximal tubule cells were similar to control and EP group (D). Bar scale: A, B, C and D is 1 μm, 2 μm, 0.5 μm and 2 μm, respectively

ative alterations in the glomerules, mesangial matrix and tubules of the kidney tissue [26].

GBM thickness increases from 6 months after diabetes induction, and its long-term increase in diabetic rats has been previously reported by Lerco *et al*. [28]. Our study lasted for two weeks when compared with the study of Lerco *et al*., which had lasted six months. In our study there was no significant decrease in the thickness of basal membrane at Diabetic+ EP group, which led us to think whether non treatment of GBM thickenning totally with EP may be the effect of long lasting complication of STZ induction. Thus, in the later studies to be carried out, long lasting experimental inducing diabetes, and together with treatment with EP must be considered to understand whether EP treats the GBM thickening totally [28].

Our data suggest that the diabetic state caused significant structural changes in glomerular barrier, which consists of GBM, podocytes and slit diaphragm. If we do not explore the antioxidants for reversible or irreversible on tissue alterations, these changes contribute to progressive glomerulosclerosis. As previously described, oxygen-derived free radicals are constantly formed in the body during normal metabolic processes. When free radical formation is greatly increased, or protective antioxidant mechanisms compromised, a state of oxidative stress is occurs. If oxidative stress persists, it will eventually lead to molecular damage and tissue injury [33]. As a result, oxidative stress has been described to be a disturbance in the balance between the production of free radicals (ROS) and antioxidant defenses, which is likely to lead to tissue injury [34]. Subjects with diabetes may be especially prone to oxidative stress, which enhances the development and progression of diabetic micro and macrovascular complications [35].

Almost all of the animal and human studies as well as in vitro experiments suggest a role of oxidative

stress through an increased formation of free radicals in the pathophysiology of diabetic microvascular complications such as nephropathy and retinopathy [35].

The human body has a multiplicity of different antioxidant defense mechanisms [36]. If the defensive processes are overwhelmed, free radicals can then become highly destructive to cells and tissues. During oxidative stress, the prooxidant-antioxidant balance is tipped in favour of the former, and this may be due to exogenous sources of free radicals or other endogenous stress [37]. However, oxidative stress can produce major interrelated dearrangements of cell metabolism, including DNA damage, protein damage and peroxidation of lipids [36].

Lipid peroxidation is known as the free radical oxidation of polyunsaturated fatty acid in biological systems reported by Gutteridge [38]. A polyunsaturated fatty acid contains two or more double bonds, and the presence of an increasing number of double bonds in fatty acids makes it more susceptible to oxidative damage by free radicals and peroxidation [33]. Furthermore, while lipid peroxidation reactions occur, cleavage of the carbon bonds causes formation of aldehyde products such as cytotoxic alkanals, alkenals and alkanes. Rice-Evans and Burdon reported that the breakdown products of lipid peroxidation, for example alkanals such as malondialdehyde (MDA), and hydroxyl alkenals such as 4-hydroxynonenal (HNE) [37] have all demonstrated cytotoxic properties [36].

In the present study, MDA activity was measured in renal tissue to evaluate the changes of antioxidant status in the kidney. Induction of diabetes in diabetic groups significantly increased in kidney tissue levels of MDA activity when compared with the control group. Ethyl pyruvate treatment in diabetic rats (DM+EP) independently produced significant decreases in MDA activity when compared with group DM. Additionally, Okutan *et al*. [39] reported that MDA content was reduced in the cardiac tissues of diabetic rats after treatment with caffeic acid phenethyl ester. These investigators have concluded that diabetes increases oxidative stress in cardiac tissue, and that caffeic acid phenethyl ester has an ameliorating effect on the oxidative stress via its antioxidant property. Except for tissue altering in the study carried out Okutan et al. and ours, the results of our study were similar to those reported by Okutan *et al.* [39] so that STZ induced diabetes increased oxidative stress; otherwise, antioxidant EP would have ameliorating effect on kidney tissue. Our findings also support those of Okutan *et al*. [39] in that MDA levels in diabetic group which had not received EP were determined to higher than the diabetic group receiving EP.

Conclusions

As the authors, we have concluded that ethyl pyruvate improves pathological alterations of the diabetic nephropathy. Additionally, it is also concluded that a detailed molecular investigation should be carried out to clarify the effects of ethyl pyruvate on diabetic nephropathy. Nonetheless, to assess the efficiency of such a new therapeutic approach in the prevention and treatment of diabetic nephropathy, long-term and large series of studies of intervention are required.

The authors declare no conflict of interest.

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