Original paper

The potential beneficial effects of ethyl pyruvate on diabetic nephropathy: an experimental and ultrastructural study

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Oxidative stress is one of the main causes of diabetic nephropathy, which is a complication of 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) of male Wistar albino rats were used as follows: control group rats received only sodium citrate buffer solution intraperitoneally (ip). The EP group was given 50 mg/kg EP ip. In the DM group, diabetes was induced by streptozotocin. The 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 was used 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 the DM group, there was evident basement membrane thickening and enlargement of mesangial matrix; swelling in some tubular epithelial cells was also noticeable. In the DM+EP administered group, nearly the same appearance as the control group and relative thickening in the glomerular basal membrane were observed. The antioxidant effect of ethyl pyruvate improved the renal structures in the DM + EP group.

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

Introduction

Diabetes mellitus (DM) is a widely occurring endocrine disorder. It is characterised by hyperglycaemia and susceptibility to chronic complications affecting the kidney (diabetic nephropathy), vasculature and eyes (diabetic retinopathy) [1]. Diabetic nephropathy is characterised by renal hypertrophy and extracellular matrix accumulation, as well as glomerular sclerosis which gives rise to proteinuria and renal failure. The precise etiological factors underlying the pathophysiological mechanism of diabetic nephropathy (DN) still remain unclear. It has been suggested that hyperglycaemia plays an important role in the pathogenesis of diabetic nephropathy by means of producing oxidative stress and advanced glycation end-products (AGEs) [2, 3].

Accumulated evidence has implicated three major pathways in the development of diabetic nephropathy in the last two decades [4]. One of them is formation of advanced glycation end-products (AGEs) by hyperglycaemia, which induces dysfunction of glomerular cells and activation of macrophages [5, 6]. The receptor of 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 either depends on oxidative stress or is secondary to oxidative stress in DM patients and a rat model of DM. Free radicals lead to tissue damage by nuclear factor κB (NF-κB). It was shown that NF-κB plays a key role in the formation of DN and iNOS activation [8, 9]. Several studies have reported that NF-κB is involved in the pathophysiological mechanism of DN. In addition, it has been shown that 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 suggested that ethyl pyruvate mediated inhibition of NF-κB activation is likely to involve more than one antioxidant effect [10, 11].

Antioxidants are molecules that combat oxidative stress and inflammation. Vitamins (C, E), resveratrol, ginseng, green tea and ethyl pyruvate are some of the important antioxidants [12, 13]. 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 streptozotocin-induced diabetes in rats' testes [22] and liver [23]. EP has been determined to be effective as pyruvate. In addition, it has been suggested to scavenge phenoxy radicals. However, the biological action of this molecule in free radical scavenging is still unknown [11].

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

Material 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 Centre of Dicle Faculty of Medicine (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 the Ethics Review Committee for Animal Experimentation, Dicle University, Faculty of Medicine, and carefully followed the guidelines "Protection of Animal Rights" during our experimental process established by NIH. Prior to the study, body mass and blood glucose levels of rats were measured.

Experimental procedure

Experimental diabetes in rats was induced by intraperitoneal application of 45 mg/kg of streptozotocin (STZ) (Sigma, USA) that had previously been 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 applied 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 in 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 ip application; Group 2: ethyl pyruvate (EP) group (n = 8) rats received 50 mg/kg EP pi 12 h at a dosing interval for totally 14 days; Group 3: DM group (n = 8), diabetes was induced by STZ ip; Group 4: DM+EP group (n = 8), diabetes was induced by STZ and rats also received 50 mg/kg EP ip 12 hours at a dosing interval of 14 days by ip 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 of 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 sacrificed under ketamine HCl (100 mg/kg, intramuscular – i.m.) and xylazine (15 mg/kg, i.m.) anaesthesia by opening the thorax wall and making an incision on the heart. The kidneys were removed: the left kidneys of the rats were used 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 buffer formalin solution. After 24 hours of fixation, the tissues were dehydrated in a 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 routine hematoxylin-eosin staining for

Table I. MDA levels of kidney tissue

GROUP	MDA* NMOL/G
Control	131.9 ±8.2
EP	139.5 ±5.9
DM	225.0 ±23.0[a]
DM + EP	167.1 ±13.5[b]

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

examination of morphologic structure of the kidney and periodic acid-Schiff stain for examination of the thickness of the proximal tubule basal membrane. The samples were examined under a BH2 Olympus light microscope. For electron microscopic examination, the biopsy samples were fixed in 2.5% glutaraldehyde solution, then postfixed with osmium tetroxide, dehydrated in increasing alcoholic solutions and embedded in Epon resin. Ultrathin sections cut by an ultramicrotome were stained with lead citrate and examined by electron microscopy (Jeol JEM 1400, Japan).

Analysis of malondialdehyde

The spectrophotometric method which was used before by Buege-Aust was used to determine the MDA tissue levels. The MDA results are reported as nmol MDA/gram tissue [25].

Statistical analysis

The program SPSS 11.0 (Chicago, IL, USA) was used for statistical analysis. The results are shown as mean ± standard deviation. Kruskal-Wallis one-way analysis of variance test was used in the analysis of the histopathological measurements and MDA levels. The Mann-Whitney U test was applied in case of a positive significant result to compare two 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 used in all statistical analyses.

Results

MDA levels

The MDA results of all groups are shown in Table I. The MDA level in the diabetic group was found to be significantly higher when compared to the control group and EP group ($p \leq 0.001$). MDA level in the 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 microscope, 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 Bowman's space, enlargement of mesangial matrix and cell proliferation. Swelling in some tubular epithelium cells which showed hydropic changes, besides the paleness of cytoplasm, and the accumulation of glycogen in various diameters and size in reaction with PAS (+) in the tubular epithelium cells of the corticomedullary area were noticeable (Fig. 1C). In the DM+EP administered group, nearly the same appearance as the control group was observed despite hydropic degeneration at a minimal level in the tubular epithelial cells as well as relative thickening in the parietal layer of Bowman's capsule and thickening of the basement membranes of tubules (Fig. 1D).

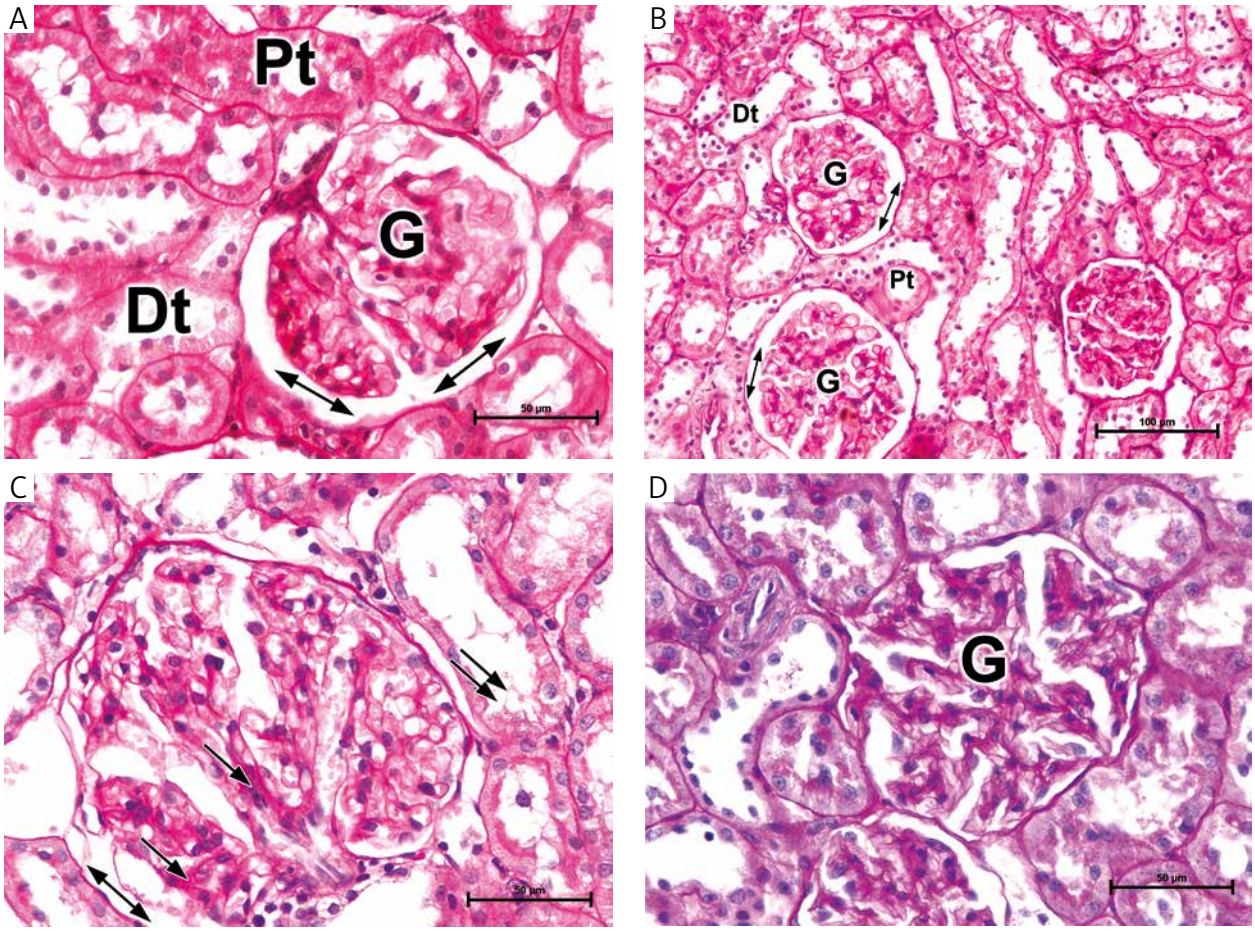
Transmission electron microscopic analysis

In the ultrathin sections of the control group, the podocyte nucleus of the kidney filtration membrane, pedicels, filtration splits, fenestrations 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 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 cisternae and tubules in DM group sections (Fig. 3A). It was detected that microvilli of proximal tubule cells had lost continuity because of degeneration and also partly enlargements in the tubular basal membrane folds were seen in another section of the DM group. At the conclusion of STZ administration, the regular dispersion of mitochondria in the basal folds was broken down, and the occurrence of autophagic vacuoles was determined (Fig. 3B).

In the DM + EP group, the ultrastructural appearance of the glomerulus, nucleus of the podocytes, pedicels and fenestrated capillaries were nearest to



Dt – distal tubule; Pt – proximal tubule; G – glomerulus; bidirectional arrows – Bowman's space; arrows – basement membrane; two arrows – hydropic changes with PAS staining.

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

normal; however, the thickening was present partly in some glomerular basement membranes (GBM) (Fig. 3C). Also in the ultrathin sections of this group, the epithelial cells of the proximal and distal tubules were found to be similar to those in the control group (Fig. 3D).

Discussion

Diabetes mellitus is considered a worldwide public health problem, both in terms of the number of people affected and premature mortality and the costs involved in controlling it and treating complications. It has been reported that DN occurs in approximately 30-40% of diabetes mellitus type 1 (DM-1) [26] cases and is the major cause of death in diabetics. 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 glycaemic levels [28, 29]. In addition to the haemodynamic effects, several non-haemodynamic factors have been identified to be involved in the pathogenesis of DN [27].

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 an advanced glycation end-product, and 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 effect of ethyl pyruvate on other organs (brain, liver, intestine) and studies about experimental diabetic rats with other antioxidants. Otherwise, there have been very few studies on ethyl pyruvate with experimental diabetic nephropathy; therefore,

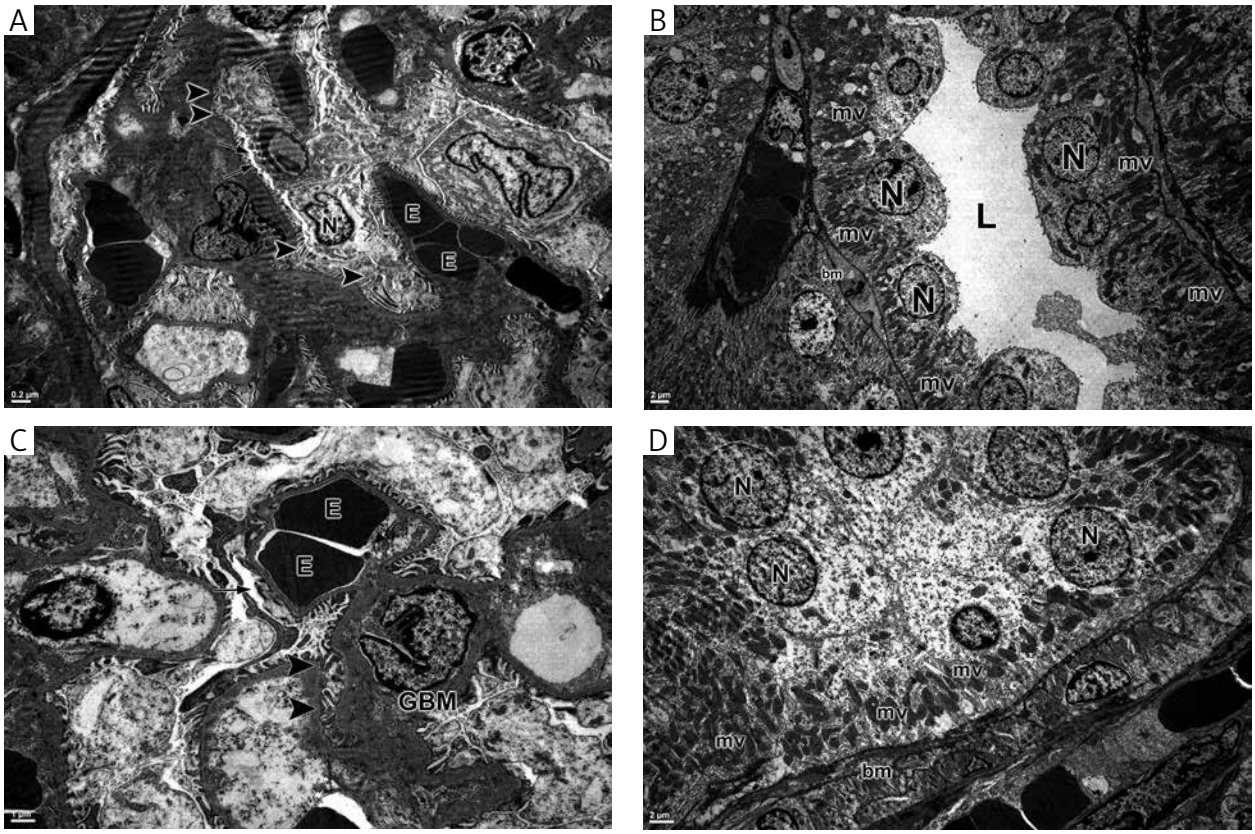


Fig. 2. Panoramic appearance of glomerular structure (A) as well as distal tubule cells (B) of cortex of kidney of the control group. Normal view of the glomerular structure (C) and distal tubules (D) of the EP group is seen. Bar scale: A, B, C and D are 0.2 μm, 2 μm, 1 μm and 2 μm, respectively

we studied 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 of tissue factor, plasminogen activator inhibitor-1, tumour necrosis factor (TNF) and tissue plasminogen activator, which are believed to have destructive effects in sepsis, was 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 some time. In STZ-induced diabetic rats, mesangial 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 observed 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 is one of the earliest alterations seen during the development of DN. Glomerular hypertrophy was seen to be exact within 4 days after diabetes was induced in rats [26]. Morphometric analysis of glomerular size showed that glomerular size increased markedly in untreated diabetic rats [32]. Through histological analysis, the expansion of glomeruli has been revealed 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 all treatment groups in terms of renal functional assessment. Thymoquinone slows down the histopathological alterations and deterioration. Irbesartan corrected the histopathological alterations more prominently than thymoquinone.

In the present study, we observed a significant decrease after the treatment of EP, so that the glomerular size, amounts of mesangial matrix and tubular 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 only reduced but did not treat the negative alterations in the glomeruli, 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

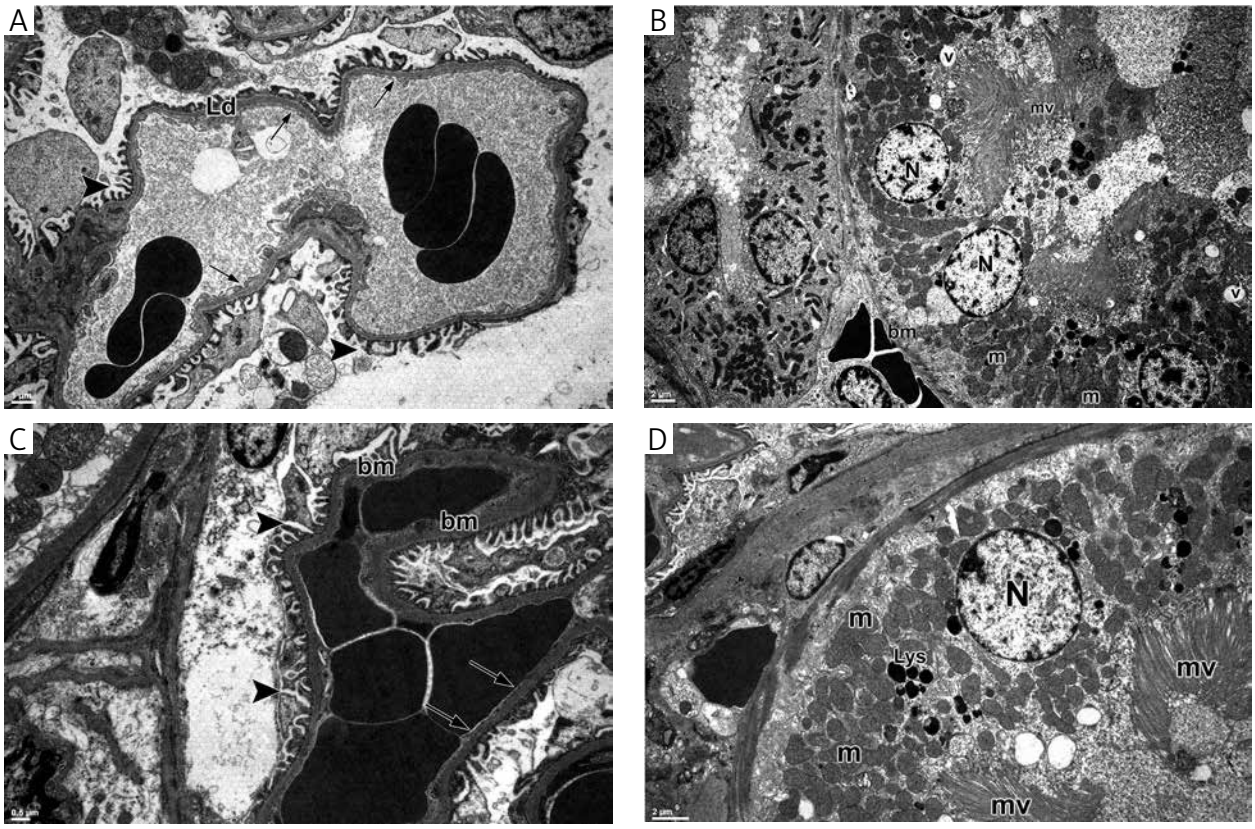


Fig. 3. Thickening of the lamina densa layer of the basement membrane is evident in glomerular structure and obliterations in slits between pedicels and capillary fenestrations (A). Additionally, degenerative changes in the proximal tubule cells with irregular shaped microvilli, and dispersing mitochondria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

rats has been previously reported by Lerco *et al*. [29]. Our study lasted for two weeks, compared with six months in the study of Lerco *et al.* [29]. In our study there was no significant decrease in the thickness of the basal membrane in the DM + EP group, which led us to wonder whether non-treatment of GBM thickening totally with EP may be the effect of a long lasting complication of STZ induction. Thus, in the later studies to be carried out, long lasting experimentally induced diabetes, 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 the glomerular barrier, which consists of GBM, podocytes and the slit diaphragm. If we do not explore the antioxidants for reversible or irreversible 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 will result. 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 (reactive oxygen species, ROS) and antioxidant defences, 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 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 defence mechanisms [36]. If the defensive processes are overwhelmed, free radicals can then become highly destructive to cells and tissues. During oxidative stress, the pro-oxidant–anti-oxidant 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 derangements 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, when lipid peroxidation reactions occur, cleavage of the carbon bonds causes formation of aldehyde products such as cytotoxic alkanals, alkenals and alkanes. Rice-Evans CA 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 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 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 alteration in the study carried out by Okutan *et al*. and in 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 an ameliorating effect on kidney tissue. Our findings also support those of Okutan *et al*. [39] in that MDA levels in the diabetic group which had not received EP were determined to be higher than the diabetic group receiving EP.

Conclusions

We conclude that ethyl pyruvate improves the pathological alterations of diabetic nephropathy. Additionally, we conclude 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 interventional studies are required.

The authors declare no conflict of interest.

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