# Protective effect of ethyl pyruvate on liver injury in streptozotocin-induced diabetic rats

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## Abstract

***Background and aims :*** **Diabetes Mellitus, leading to an increase in oxidative stress, can cause liver damage. Our aim was to investigate the antioxidant effects of Ethyl Pyruvate (EP) on the liver tissue in diabetic rats.**

***Materials and methods :*** **Thirty-two Wistar albino rats were separated into four equal groups. Groups were assigned as follows : (1) Non-diabetic group ; (2) EP-treated non-diabetic group ; (3) diabetic group ; and (4) EP-treated diabetic group. In order to induce diabetes mellitus, 45 mg/kg b.w. streptozotocin was administered intraperitoneally to the rats in groups 3 and 4 . On the 3rd day, blood glucose was assessed. Rats with blood glucose levels higher than 300 mg/dl were considered to be diabetic. The EP solution was administered intraperitoneally at a dose of 50 mg/kg b.w. twice daily for 14 days to the rats in groups 2 and 4. The other rats were simultaneously given the same amount of Ringer's lactate solution intraperitoneally. Liver tissue was obtained for malondialdehyde (MDA) analyses and histopathological examination.**

***Results :*** **In group 4, Total Antioxidant Status (TOS) and MDA levels were significantly lower as compared to group 3 . Also, morphological abnormalities occurred in group 3 when compared with non-diabetic groups (groups 1 and 2), whereas the disorders resulting from diabetes improved significantly in group 4.**

***Conclusions :*** **These findings show that EP has protective effects against diabetes-induced liver injury.** (Acta gastroenterol. belg., **2012**, 75, **336-341**).

**Key words** : Diabetic liver injury, Ethyl pyruvate, Streptozotocin, Oxidative stress.

## Introduction

Diabetes mellitus (DM) is quite a common metabolic disease that can result in severe structural and functional complications in liver (1,2). In addition to unfavourable effects on many tissues and organs, it has been suggested that DM also adversely affects hepatobiliary functions in diabetic patients and animals. Further, it is reported that hepatobiliary disorders such as hepatic inflammation, non-alcoholic fatty liver disease, nonalcoholic steatohepatitis, hemochromatosis, autoimmune hepatitis, cirrhosis, hepatocellular carcinoma, acute liver failure, and cholelithiasis can ensue under diabetes (1-3).

Studies have shown that high glucose concentrations lead to oxidative stress caused by impairment of mitochondrial electron transfer and activation of the polyol pathway resulting in the excessive generation of reactive oxygen species (4,5). In vitro experimental studies as well as studies on humans and animals have demonstrated the role of increased oxidative stress in the development of diabetic complications (6,7). Oxidative stress contributes to the development of complications in diabetic individuals by causing functional and structural changes in the cell membrane and subcellular molecules (7,8). Several studies have also shown that antioxidant treatment is beneficial in diabetic rats and in diseases associated with oxidative stress (3,9-11).

Pyruvate is the trivial name for 2-oxoproprionate, $CH3COCOO^-$. Pyruvate is the product of the reaction catalyzed by the enzyme, pyruvate kinase, which is the last step of the glycolytic pathway. Pyruvate is also produced by a number of other biochemical reactions in mammalian cells, such as the transamination of alanine and a-ketoglutarate by alanine aminotransferase. Under anaerobic conditions, much of the pyruvate generated by glycolysis is reduced in the lactate dehydrogenase reaction to form lactate (i.e., 2-hydroxyproprionate) and the oxidized form of nicotinamide adenine dinucleotide ($NAD^+$). Under aerobic conditions, pyruvate is transported from the cytosol into mitochondria where it is oxidized to form acetyl coenzyme A by the enzyme complex, pyruvate dehydrogenase (12).

Pyruvate, a small molecule normally regarded as a key intermediate in anaerobic and oxidative glucose metabolism, is also a potent and effective reactive oxygen species (ROS) scavenger (13). After the discovery of the free radical scavenger effect of pyruvate, researchers have implicated this molecule in the treatment of ischemia-reperfusion injury (14-16), hemorrhagic shock (17), stroke (18), diabetic cataract (19), testicular torsion (20), extrahepatic cholestasis (21) as well as many other pathological conditions (22). Although positive effects of pyruvate have been reported in these studies, the usefulness of pyruvate as a therapeutic agent is limited by its poor stability in solutions. In order to overcome this limitation, its lipophilic ethyl ester form, ethyl pyruvate (EP), is being used in clinical practice (22).

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Antioxidant effects of pyruvate appear to have been extensively demonstrated in different conditions where involvement of oxidative stress is suspected (14-21). Until now, perhaps, the modified form of pyruvate (i.e., EP) has not yet shown similar protection against liver damage due to DM. The present study aimed to investigate the effects of EP on the liver injury in streptozotocin-induced diabetes rats.

## Materials and methods

Thirty two Wistar albino male rats aged between 7 and 8 weeks and weighing between 200 and 240 g were used in the present study. In the course of experiment, the rats were kept in a 12 h dark/12h light cycle room having temperature 23 ± 2°C and constant relative humidity (60%), and were maintained on standard laboratory diet and water ad libitum. The study was initiated after obtaining approval from Dicle University Local Committee on Animal Research Ethics.

### *Experimental Method and Procedure*

STZ (Sigma, USA) was used to induce diabetes in rats (23). A solution of STZ was freshly prepared in citrate buffer (pH : 4.5) and was administered intraperitoneally at a dose of 45 mg/kg b.w. to diabetes induced rats. Citrate buffer solution of pH : 4.5 was simultaneously injected intraperitoneally to the rats in the non-diabetic group. Seventy-two hours after STZ administration, blood glucose levels of the rats were measured with a Medisense Optium glucometer (Roche, Germany) using the blood obtained from the tail vein. Rats with a blood glucose level ≥ 300 mg/dL were considered diabetic.

Fourteen weeks after the induction of diabetes, diabetic and non-diabetic rats were assigned into two groups each containing 8 rats. The groups were as follows : (1) Non-diabetic group ; (2) EP-treated non-diabetic group ; (3) diabetic group ; and (4) EP-treated diabetic group. Sixteen rats in group 3 and 4 were splited by ≥ 300 mg/dL glucose threshold.

EP (Sigma, USA) was dissolved in Ringer's lactate solution containing 130 mmol/L $Na^+$, 4.0 mmol/L $K^+$, 2.7 mmol/L $Ca^{++}$, and 109 mmol/L $Cl^-$ at pH 7.0 (24). The EP solution was administered intraperitoneally to the rats in the EP-treated diabetic and non-diabetic groups at a dose of 50 mg/kg b.w. twice daily for 14 days. The other rats were simultaneously given the same amount of Ringer's lactate solution intraperitoneally.

The rats were sacrificed on the 15$^{th}$ day by cardiac puncture under ketamine and xylazine anesthesia. Venous blood samples were collected. Following this process, their livers were removed for histological examination purposes.

### *Malondialdehyde Analysis*

MDA levels in the liver tissues were determined spectrophotometrically by using the method described by Buege and Aust (25). For this purpose, a UV-1205 Shimadzu spectrophotometer was used to determine absorbance at 532 nm. The results were expressed as nmol of MDA/g tissue.

### *Total Antioxidant Status, Total Oxidant Status and Oxidative Stress Index*

Total oxidant status (TOS) and Total antioxidant status (TAS) were measured in supernatant fraction of homogenates and serum samples a commercially available Rel Assay Diagnostic kits with an autoanalyzer (Architect c16000). TOS results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (mmol $H_2O_2$ equivalent/L) (26), and TAS results were expressed as mmol Trolox equivalent/L (27). The ratio percentage of the TOS to the TAS potential gave the oxidative stress index (OSI), an indicator of the degree of oxidative stress (28).

### *Histopathological Examination*

All tissue specimens were obtained from the central part of the right lobe of the liver and were fixed in 10% neutral buffered formalin solution, processed for embedding in paraffin by routine protocols, and 5μm thick sections were then cut by microtome. The sections were stained with Hematoxylin–Eosin by using a routine protocol and examined with a Nikon Eclipse 80i photomicroscope. The pathological findings of examination by using light microscopy were scored as 0 (no observed changes), 1 (mild changes), 2 (moderate changes), or 3 (severe changes) and were assessed in a blinded manner (3).

For electron microscopy, liver tissue specimens were fixed overnight in 2.5% glutaraldehyde (pH : 7.3) in 0.1 M sodium phosphate buffer. The tissues were postfixed in 2% osmium tetroxide and then dehydrated through a graded series of ethanol. The tissue specimens were embedded in araldite. The araldite blocks were sectioned using a ultramicrotome (Ultracut R Leica, Germany). Ultrathin sections were stained with uranyl acetate and subsequently with lead citrate ; they were examined and photographed by using a transmission electron microscope (Jeol TEM 1011, Japan).

### *Statistical Analysis*

Statistical analysis was conducted by using the Statistical Package for the Social Sciences for Windows (version 11.0 ; Chicago, USA). TOS, TAS and OSI results were expressed as means ± standard deviation, and Kruskal-Wallis test was used for analysis. In the event of significant results, the Mann-Whitney U test was used for comparisons of differences between two independent groups. The Mann-Whitney U test was also used to compare non-diabetic and diabetic rats in terms of body weight and blood glucose levels. Histopathological results were expressed as median values and analyzed by Kruskal-Wallis test. A p value $< 0.05$ was considered statistically significant.

Table 1. — **Levels of MDA, TOS and TAS and the OSI in different groups**

| Group | MDA* nmol/gram tissue | TOS* mmol $H_2O_2$ /L | TAS mmol Trolox /L | OSI* |
|---|---|---|---|---|
| N-DM (n :8) | 161.8 ± 26.1 | 2.94 ± 0.78 | 0.49 ± 0.13 | 6.19 ± 2.04 |
| EP (n :8) | 164.81 ± 9.5 | 3.04 ± 0.55 | 0.47 ± 0.12 | 6.77 ± 2.26 |
| DM (n :8) | 252.8 ± 18.0[a] | 5.47 ± 0.92[a] | 0.37 ± 0.10 | 15.74 ± 5.77[a] |
| DM+EP (n :8) | 207.9 ± 30.8[b] | 3.87 ± 1.74[c] | 0.44 ± 0.07 | 8.76 ± 3.56[c] |

Results are presented as means ± standard deviation.
*p < 0.01 for Kruskal Wallis test.
[a] p = 0.001 as compared to the N-DM and EP group , [b] p < 0.01 as compared to the DM group. [c] p < 0.05 as compared to the DM group.
N-DM : non diabetes mellitus, EP : ethyl pyruvate treated non-diabetic group, DM : diabetes mellitus, DM+EP : ethyl pyruvate treated diabetic group. MDA : malondialdehyde, TOS : Total oxidant status, TAS : Total antioxidant status, OSI : Oxidative stress index.

Table 2. — **Histopathological findings in the different study groups**
(0 : no observed changes, 1 : mild changes, 2 : moderate changes, 3 : severe changes)

| | N-DM | EP | DM | DM+EP | P* |
|---|---|---|---|---|---|
| Parenchymal cells | | | | | |
| Hydropic swelling | 0 | 0 | 2.5 | 0.5 | p < 0.001 |
| Granular degeneration | 0 | 0 | 1 | 0 | p < 0.001 |
| Microvesicular vacuole | 0 | 0 | 2 | 0.5 | p < 0.001 |
| Macrovesicular vacuole | 0 | 0 | 2 | 0 | p < 0.001 |
| Focal necrosis | 0 | 0 | 0 | 0 | p > 0.05 |
| Cordon plan disarrangement | 0 | 0 | 3 | 1 | p < 0.001 |
| Portal area | | | | | |
| Inflammation | 0 | 0 | 3 | 1 | p < 0.001 |
| Fibrosis | 0 | 0 | 0 | 0 | p > 0.05 |
| Sinusoids | | | | | |
| Hyperemia | 0 | 0 | 3 | 2 | p < 0.001 |

* DM group was compared with the other groups.
N-DM : non diabetes mellitus, EP : ethyl pyruvate treated non-diabetic group, DM : diabetes mellitus, DM+EP : ethyl pyruvate treated diabetic group.

## Results

On the last day of the study, the mean body weight and the mean blood glucose level of the rats in the diabetic group were 214 ± 3.7 g and 450 ± 13.6 mg/dL, respectively ; the mean body weight and the mean blood glucose level of the rats in the non-diabetic group were 303 ± 7.1 g and 147 ± 4.6 mg/dL, respectively. As compared with the non-diabetic group, the mean body weight of the rats in the diabetic group was significantly lower (p < 0.001), whereas the mean blood glucose level was significantly higher (p < 0.001).

### *Mean Levels of TOS, TAS and the OSI*

Mean levels of TOS and TAS and the OSI are shown in Table 1. TOS and OSI levels were significantly higher in the diabetic group as compared to the non-diabetic and EP-treated non-diabetic groups (p = 0.001). TOS and OSI levels of the EP-treated diabetic group were significantly lower as compared to the diabetic group that did not receive EP (p < 0.05). TAS level was not significantly different between groups (p > 0.05).

### *Malondialdehyde Levels*

MDA levels of the groups are shown in Table 1. MDA level was significantly higher in the diabetic group as compared to the non-diabetic and EP-treated non-diabetic groups (p = 0.001). MDA level of the EP-treated diabetic group was significantly lower as compared to the diabetic group that did not receive EP (p < 0.01).

### *Histopathological Results*

The histopathological findings are shown in Table 2. In H-E stained slides, there was no focal necrosis of parenchymal cells and fibrosis in portal area in all groups (Fig. 1a-d). Furthermore, normal characteristic features of liver were apparent in non-diabetic and EP-treated non-diabetic groups (Fig. 1a-b). Histopathological alterations that were observed in diabetic rats, such as hydropic swelling, granular degeneration, hepatocytes

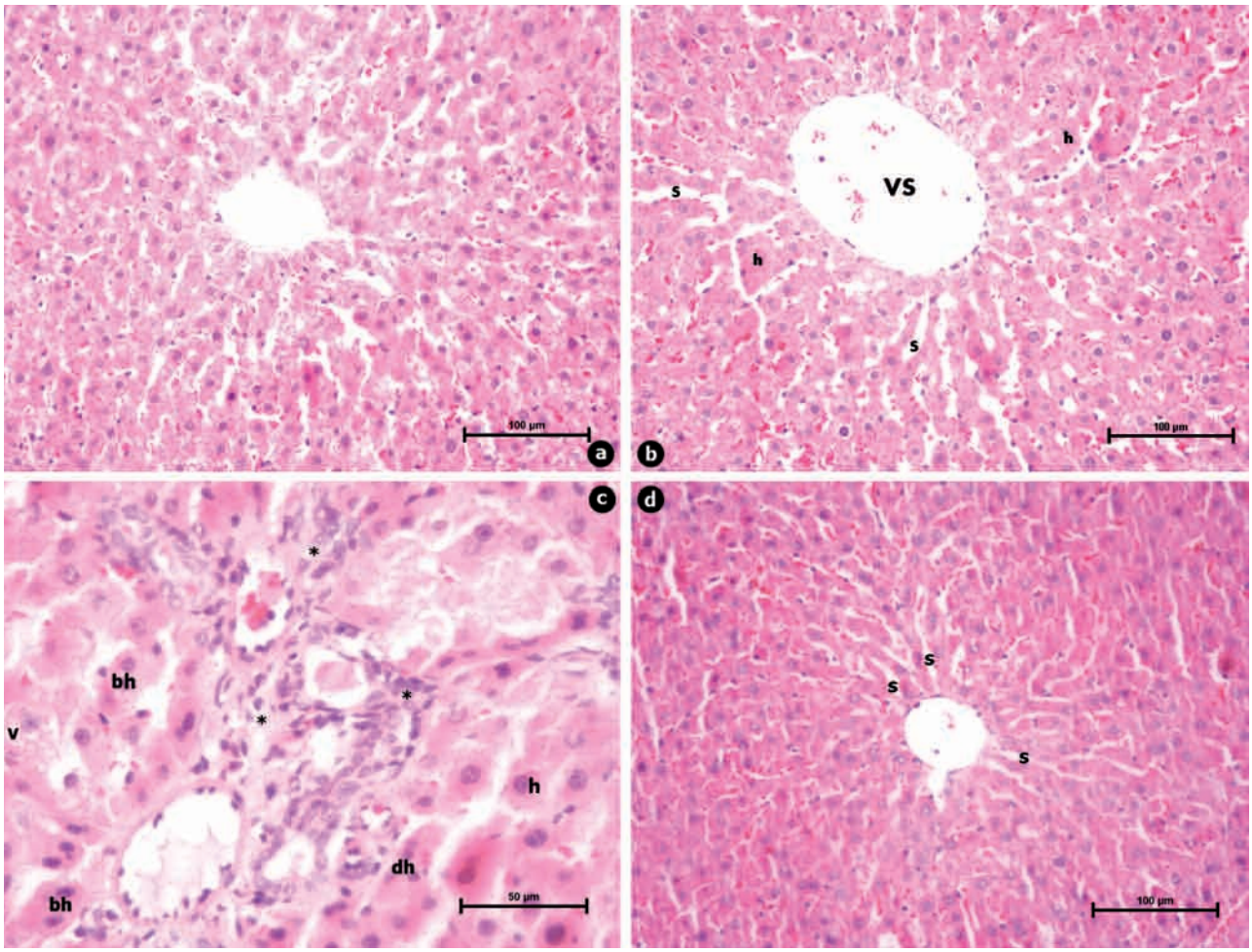


Fig. 1. — Photomicrographs of Hematoxylin and Eosin stained sections of liver of rats (a) Typical features of normal appearance of liver tissue are observed in non-diabetic group (b) vena centralis (vs), radial arrangement of hepatocytes (h) and sinusoids (s) were observed in ethyl pyruvate treated non-diabetic group c) increased number of binucleate hepatocytes (bh), inflammation in portal area (*), microvesicular vacuolisation (v) and disarrangement of hepatocytes (dh) plate are observed in diabetic group d) Most of the alterations occurring in liver of diabetic rats were prevented by ethyl pyruvate ; except dilation of sinusoids ethyl pyruvate treated diabetic group.

trabeculae disarrangement, microvacuolization, macrovacuolization, and inflammation (Fig. 1c) were significantly improved in EP-treated diabetic group (Fig. 1d).

Electron microscopic examination of the hepatocytes in non-diabetic group (Fig. 2a) and EP-treated non-diabetic group (Fig. 2b) livers showed structurally normal nuclei and cell organelles. However, degenerative changes, such as mitochondrial swelling and crystolysis, dilation of rough endoplasmic reticulum, increase of autophagic vacuoles and lipid granules, were detected in the hepatocytes of the diabetic rats. Also, there were no glycogen deposits in the cytoplasm of these cells (Fig. 2c). The signs of degeneration observed in diabetic group samples were apparently less in the EP-treated diabetic group. Rough endoplasmic reticulum and mitochondria were arranged in a regular manner and glycogen deposits were detected in the cytoplasm of hepatocytes in the EP-treated diabetic rats (Fig. 2d).

## Discussion

Oxidative stress resulting from the imbalance between free radical generating and scavenging systems (29) has been involved in the pathogenesis of many tissue complications in diabetes mellitus (30). In our study, morphological alterations were observed in streptozotocin-induced diabetic rats liver. These signs were considered to be indicators of oxidative damage in liver. The aim of the present study was to evaluate protective effects of ethyl pruvate on liver damage in rats that lived with DM for 14 weeks.

DM leads to irregularity of carbohydrate, protein and lipid metabolism as well as increase in lipid peroxidation. Increase in lipid peroxidation and oxidative stress plays an important role in the development of diabetic complications (31). MDA is a product of lipid peroxidation which increases in cells and body fluids (32,33). Increase

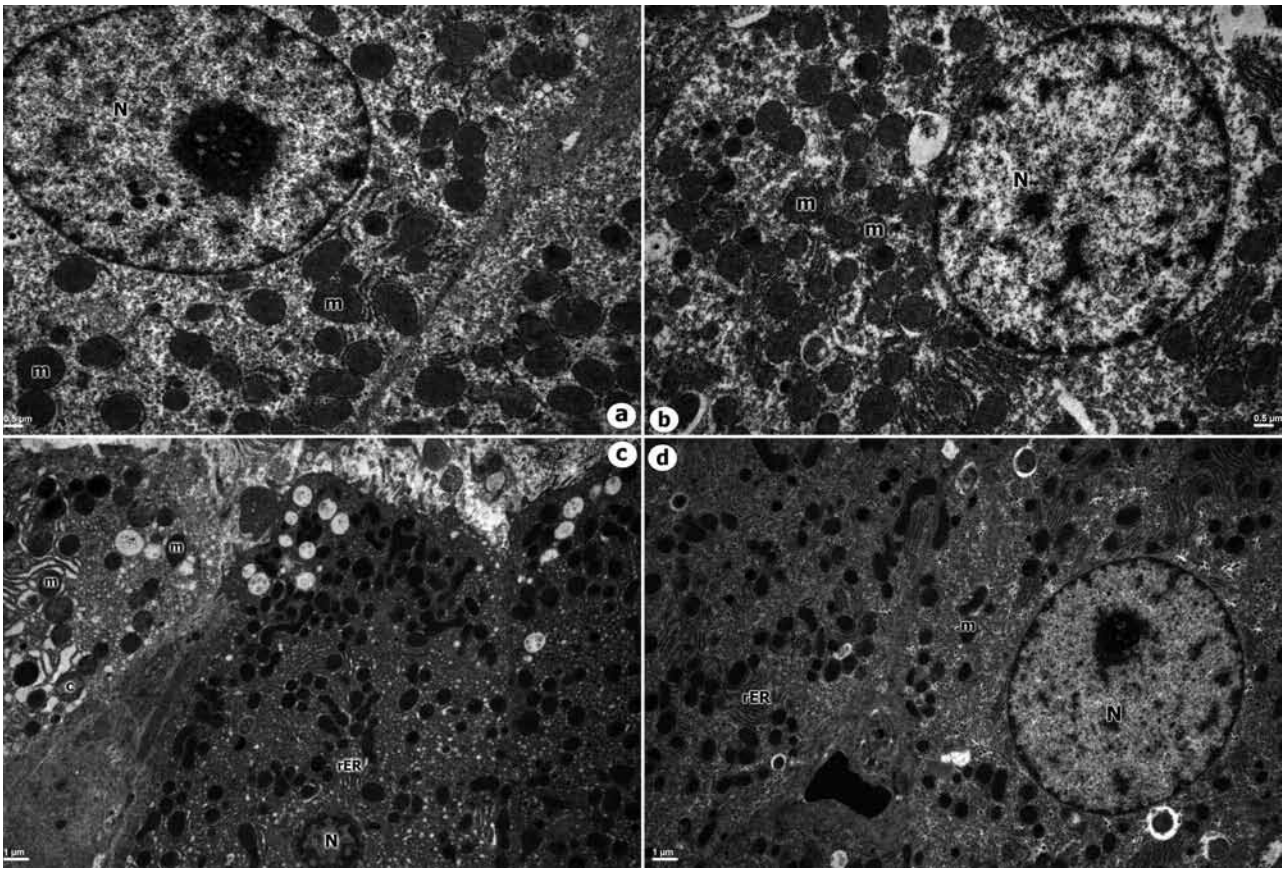


Fig. 2. — Electron micrographs of the hepatocytes a) Ultrastructural typical features of liver appearance are observed in non-diabetic group b) An electron microscopic viewing of hepatocytes is seen in ethyl pyruvate treated non-diabetic group, mitochondria (m), nucleus (N). c) Loss of glycogen, mitochondrial (m) swelling and crystolysis (c) as well as increase in lipid granules, autophagic vacuoles and dilation of rough endoplasmic reticulum (rER) are observed in diabetic group d) Degeneration of hepatocytes were prevented ; the rER and mitochondria (m) were observed in normal appearance in ethyl pyruvate treated diabetic group.

in lipid peroxidation levels can be determined by measuring MDA levels.

Pritchard *et al*. reported that vitamin E decreases plasma and liver levels of MDA in streptozotocin-induced diabetic rats (34). In several studies it was reported that diabetes not only increased MDA levels in liver but also kidney, heart and brain. These studies suggest that antioxidants therapy can decrease MDA levels, as well (35,36). In this study, MDA levels were significantly higher in the diabetic group compared to the non-diabetic group. However, it was observed that the increase was reduced in the rats treated with EP. This finding indicates that diabetes-induced lipid peroxidation is likely to be improved with EP administration.

In the present study, we assayed oxidative status as TOS and TAS along with the calculation of OSI, an indicator of oxidative stress, which reflects the redox balance between oxidation and antioxidation (37). Since separate measurement of different oxidant molecules such as superoxide radical anion, hydrogen peroxide is not practical and their oxidant effects are additive, we measured TOS in serum as previously described by Erel (26). Likewise, we measured TAS, instead of measuring antioxidant molecules separately following the methods of Erel (27) and Cikrikcioglu *et al*. (38). Recently, it has been reported that OSI may reflect the oxidative status more accurately than TOS or TAS level alone (28,39). Horoz *et al*. showed relationship between plasma levels of TAS-TOS and non-alcoholic fatty liver disease (40). The results of this study showed that oxidative stress was increased in these patients. In a study performed on patients with non-alcoholic fatty liver disease, plasma levels of TAS and TOS were measured, and researchers reported that oxidative stress increased due to steatohepatitis and these signs are reliable and applicable for antioxidant level of organism (40). In addition, similar results have been shown in a study on hepatitis B virus infected patients (39). In the present study, no significant difference was observed between the groups in terms of TAS level. The levels of TOS and OSI were significantly increased in diabetic group compared with non-diabetic groups. On the other hand, TOS and OSI levels of EP treated diabetic rats were significantly decreased compared with diabetic rats. These results showed that diabetes leads to increase in oxidative stress, and that the rising of oxidative stress was prevented by application of EP.

Both Kushwaha *et al*. (35) and Unal *et al*. (41) demonstrated that diabetes leads to vacuolization, degen-

eration of hepatocytes cytoplasm and pycnotic cells in liver sections at light microscopic level. Our findings in diabetic group were similar to the results of these studies, except pycnotic cells.

Guven *et al*. (3) reported that melatonin protects liver injury in streptozotocin induced diabetic rats. The protective efficacy of melatonin is attributed to its antioxidant impact. In accordance with this suggestion, ethly pyruvate, having an antioxidant efficacy and used in our study, was observed to have improved diabetes-induced liver injury both at light and electron microscopic examinations.

In conclusion, to the best of our knowledge, this study is the first to investigate the effects of EP against diabetes-induced liver injury in diabetic rats. EP showed protective effects against diabetes-induced liver injury. These effects, probably, result from the antioxidant properties of EP.

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