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Effects of caffeic acid phenethyl ester on oxidative stress, hystopathology and some biochemical parameters in streptozotocin-induced diabetic rats

[Streptozotocin ile indüklenen diyabetik sıçanlarda oksidatif stres, histopatolojik ve bazı biyokimyasal parametrelere kafeik asit fenetil esterin etkileri]

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ABSTRACT

Objective: Diabetes mellitus (DM) is a significant health problem and its treatmet has taken herbal and syntetic remedies as well as insulin therapy. The aim of this study was to evaluate the therapeutic effects of caffeic acid phenil ester (CAPE) in rats with streptozotocin (STZ)-induced diabetes using hystopathological and biochemical methods.

Methods: 22 adult Wistar Albino strain male rats were divided into three groups: control group (n=8), diabetic group (n=6) and the diabetic+CAPE group (n=8). Two groups were injected intraperitoneally (i.p.) with 50 mg/kg STZ to induce DM. Rats with blood glucose levels of 270 mg/dL or greater and with siagns of polyuria and polydipsia after 3 days were considered to be diabetic. Diabetic+CAPE group was given ip CAPE (10 µmol/kg/during 60 days) for treatment.

Results: Liver malondialdehyde (MDA) level was significantly higher in the diabetic group. The higher liver MDA was supported by higher enzyme levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). MDA levels in kidney were not significantly different between groups and superoxide dismutase (SOD) levels were increased in CAPE group. Additionally, blood urea nitrogen (BUN) and creatinine enzyme levels were mostly stable in serum samples. At the end of the experiment, pancreatic tissue MDA in the experimental groups decreased compared with the control group. The kidney, liver and pancreas had almost normal histological structure in the CAPE group when compared to the diabetic group. Considering blood glucose levels, CAPE treatment maintained blood glucose at the same level with controls, compared to diabetic group.

Conclusion: Liver tissue might be the most affected by oxidative stress caused DM. A single injection of STZ decreased the level of MDA in pancreatic tissue at the end of experiment. This decrease might be exhausting MDA due to disruption in tissue integrity. Consequently, it was observed that CAPE suppressed oxidative stress and decreased glucose levels in STZ-induced diabetic rats. Thus, it may be useful to use as remedies, the antioxidant properties, in addition to anti-diabetic drugs in DM.

Key Words: CAPE, Diabetes Mellitus, Hyperglysemia, Oxidative stress, Streptozotocin

Conflict of Interest: The authors have no conflict of interest.

ÖZET

Amaç: Diabetes mellitus (DM) önemli bir halk sağlığı problemidir ve tedavisinde insulin terapisinin yanısıra bitkisel ve sentetik ilaçlar verilmektedir. Bu çalışmanın amacı, streptozotocin (STZ) ile indüklenen diyabetli sıçanlarda histopatolojik ve biyokimyasal metotlar kullanılarak CAPE'nin tedavi edici etkilerini değerlendirmektir.

Metod: 22 yetişkin erkek Wistar Albino ırkı sıçan 3 gruba ayrıldı: kontrol grubu (n=8), diyabetik grup (n=6) ve diyabetik+CAPE grubu (n=8). 2 gruba periton içi (ip) 50 mg/kg STZ enjekte edilerek DM uyarıldı. Üç gün sonra poliüri ve polidipsi belirtileri ile kan glikoz seviyeleri 270 mg/dl ya da daha yüksek olan sıçanlar diyabetik olarak kabul edildi. Diyabetik+CAPE grubuna tedavi için CAPE 60 gün boyunca 10 µmol/kg dozunda ip olarak uygulandı.

Bulgular: Karaciğer malondialdehit (MDA) seviyeleri diyabetik grupta önemli derecede yüksekti. Yüksek karaciğer MDA seviyesi, yüksek alanin aminotransferaz (ALT) ve aspartat aminotransferaz (AST) enzim seviyeleri tarafından desteklendi. Böbrek dokusundaki MDA seviyesinin diğer gruplar ile arasında önemli bir farklılık yoktu ve süperoksit dismutaz (SOD) seviyeleri CAPE grubunda artmıştı. İlave olarak kan üre nitrojen (BUN) ve kreatinin düzeyleri serum örneklerinde sabit kaldı. Deneyin sonunda pankreas dokusunda MDA seviyeleri kontrol grubuyla karşılaştırıldığında deneme gruplarında düşüktü. Böbrek, karaciğer ve pankreas dokularının histopatolojik sonuçları, diyabetik grupla karşılaştırıldığında CAPE grubunda normal histolojik yapıdaydı. Kan glikoz seviyeleri göz önüne alındığında, CAPE ile tedavi edilen grupta, diyabet grubundaki yüksek glikoz seviyeleri ile karşılaştırıldığında kontrol grubuna benzer seviyelerde kan glikozu muhafaza edilmişti.

Sonuç: DM'un sebep olduğu oksidatif stres tarafından en çok etkilenen karaciğer dokusudur. Tek seferde STZ enjeksiyonu ile deneme sonunda pankreatik dokuda MDA seviyeleri azaldı. Bu azalma, doku bütünlüğünün bozulmasından kaynaklanan MDA'nın tükenmesi olabilirdi. Sonuç olarak, CAPE'nin oksidatif stresi baskıladığı ve STZ ile uyarılan DM'da kan glikoz düzeylerini düşürdüğü gözlemlendi. Sonuç olarak, DM'da antidiyabetik ilaçlara ilave olarak antioksidan özellikli ilaçları kullanmak faydalı olabilir.

Anahtar Kelimeler: CAPE, Diabetes Mellitus, Hiperglisemi, Oksidatif stres, Streptozotocin

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

Diabetes mellitus (DM), resulting in chronic hyperglycemia, is affected by many environmental and genetic factors and resulted in biochemical changes and oxidative stress [1,2]. They play a role in the symptoms and progression of the disease [3] and can result in over production of oxygen free-radical and/or decrease the efficiency of the antioxidant system [4]. There are various endogenous defence mechanisms for free radicals, including GSH, and SOD, GPx and CAT enzymes. Their activities eliminate superoxides, hydrogen peroxide and hydroxyl radicals. The oxygen free radical generation in DM has been associated with auto-oxidation of glucose [5]. Szkudelski [6] reported that oxidative stress is increased in experimental models of streptozotocin (STZ)-induced diabetes mellitus in rats. DM impaired glutathione metabolism, and caused alterations in the antioxidant enzymes and generation of lipid peroxides [7,8].

The conventional therapy for DM consists of hypoglycemic drugs and insulin [9]. Due to the side effects of this therapy, the treatment strategies have taken new directions including herbal and synthetic methods [10-12]. Caffeic acid phenethyl ester (CAPE), an active component of propolis, is one of these. The effects of CAPE (antimicrobial, anti-inflammatory, immunomodulatory, antimutagenic and antioxidant) have been revealed in several studies [13-17]. For this reason, we used CAPE in the current study. This research work aimed to investigate the antioxidant effects, on liver, kidney, and pancreatic tissue, and anti-hyperglisemic effects of CAPE in rats with STZ-induced DM after 60 days, using hystopathological and biochemical methods.

Methods

Animal model and diets

Ninety- days- old male Wistar rats weighing 225±25 g were given a normocaloric standard diet and water *ad libitum*, while being maintained in a controlled environment (12 h light and dark cycle, 21–23°C). The animals were acclimatized to the laboratory conditions for one week before the start of the experiment.

Experimental design

Twenty- two rats were divided into three groups: group I was the non-diabetic control (8 rats); group II was diabetic (6 rats); group III was diabetic+CAPE (8 rats). CAPE was administrated (10 µmol/kg/day (CAPE-Sigma) intraperitoneally (i.p.) daily for 60 days. After the last treatment, the rats were put down by cervical dislocation under anesthesia. Serum and tissue samples were taken for the evaluation of biochemical, oxidative parameters and hystopathology. The study protocol was reviewed and approved by Mustafa Kemal University Ethics Committee for Animal Research (Protocol No:2010/03/11).

Induction of experimental diabetes

Streptozotocin (STZ) was freshly dissolved in a citrate buffer (0.01 M, pH 4.5) and maintained on ice prior to use. Diabetes was induced with a single i.p. injection of STZ (50 mg/kg). Diabetic status was confirmed in the STZ-applied rats by measuring the fasting plasma glucose after 72 hours, and animals with blood glucose measuring 270 mg / dL were considered to be diabetic. Fasting blood glucose was detected by using the one touch glucometer (Accu-chek sensor) of Roche Diagnostics, Germany.

Oxidative Parameters

All tissue samples were stored at -30°C until assayed for MDA, CAT and SOD activities. The liver, pancreas and kidney tissues were cut into small pieces and homogenized (for 2 minutes at 5000 rpm) in 4 volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) using a homogeniser (ICA, T10-B, Germany).

MDA levels in the tissue homogenate were measured with thiobarbituric acid reaction by the method of Esterbauer and Cheeseman [18] as described in previous studies. The values were expressed as nmol / g protein.

The total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun *et al*. [19]. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the nitro blue tetrazolium (NBT) reduction rate. SOD activity was also expressed as units / mg protein.

CAT (EC 1.11.1.6) activity was determined according to Aebi's [20] method. The principle of the method is based on the determination of the rate constant k (dimension: s^{-1}, k) for the hydrogen peroxide (H_2O_2) decomposition rate at 240 nm. Results were expressed as k/g protein.

The protein content in liver, kidney and pancreatic tissues was measured by the method of Lowry *et al*. [21] with bovine serum albumin as standard.

Serum AST, ALT, BUN, Creatinin and glucose determinations

Serum AST, ALT, BUN, Creatinine levels were determined by spectrophotometric methods using an autoanalyser (Beckman Coulter LX-20, USA).

Fasting blood glucose was detected by using the one touch glucometer (Accu-Chek sensor) of Roche Diagnostics, Germany, in the beginning and at the end of the experiment.

Hystopathological examination

Liver, kidney and pancreas specimens were fixed in 10% neutral-buffered formaldehyde solution. After dehydration procedures, the samples were blocked in paraffin. Four micrometer sections were cut by a microtome (rotary microtome-5µm) and stained with hematoxylin and eosin. Tissue sections were examined using a light microscope (Olympus CX-31 trinocular microscopes DP20 digital camera, Japan). Evaluations were performed based on general histologic appearance and no scoring was made.

Table 1. Glucose levels of control, CAPE and diabetic groups at the beginning, the application of STZ and at the end of experiment (mean±SEM)

Glucose levels (mg/dl)	Control group (N:8)	Diabetic group (N:6)	CAPE group (N:8)	p
Begining of experiment	88.25±4.23^{ay}	73.00±4.02^{by}	71.00±2.24^{bz}	0.004
Application of STZ	88.25±4.23^{by}	472.17±45.36^{ax}	428.13±16.61^{ax}	0.000
End of experiment	187.63±10.44^{bx}	445.00±54.69^{ax}	190.00±21.58^{by}	0.000
	(x2.2)	(x6.1)	(x2.7)	
P	0.000	0.000	0.000	

a, b and x,y: different letters in the lines and columns are statistically significant (Tukey test, p<0.05). Parenthesis (x) explain xfold increase or decrease of parameters compared to control group.

Table 2. Enzyme levels of control, CAPE group and diabetic groups (mean±SEM)

	Control group (N:8)	Diabetic group (N:6)	CAPE group (N:8)	p
AST (Unit/L)	97.33±6.84^{b}	287.83±58.12^{a}	170.30±16.23^{b}	0.002
		(x2.96)	(x1.75)	
ALT (Unit/L)	25.5±1.34^{b}	88.830±27.83^{a}	45.00±3.10^{ab}	0.017
		(x3.5)	(x1.76)	
UREA (mg/dl)	31.17±0.95^{a}	48.83±8.45^{a}	43.0±3.96^{a}	0.099
		(x1.57)	(x1.38)	
CREATININE (mg/dl)	0.30±0.002^{a}	0.35±0.02^{a}	0.29±0.03^{a}	0.357
		(x1.16)	(x1)	

a, b; different letters in the same line are statistically significant (Tukey test, p<0.05). Parenthesis (x) explain xfold increase or decrease of parameters compared to control group.

Statistical analysis

Data were analyzed with a commercially available statistics software package (SPSS15 for Windows, Chicago, IL). Analysis of one-way variance (ANOVA) and the Tukey test were used for data evaluation. The results are presented as means±SEM. P values less than 0.05 were regarded as statistically significant.

Results

At the end of the 60th day, some pathological and biochemical changes were determined and given in tables and figures.

Biochemical results;

The diabetic group's blood glucose levels were higher than those of the CAPE treatment and control groups (Table 1).

The MDA level in liver tissue was higher in diabetic group compared to other groups and significant (p<0.05). On a parallel with these findings, liver enzyme levels (ALT, AST) in the serum samples were also higher in DM group (p<0.05). Changes in SOD and CAT of the CAPE group tended to increase compared to diabetes, but not significant (Tables 2 and 3).

Renal oxidative stress parameters were not significantly changed at the end of the 60 days, and the concentrations of BUN and creatinine in the serum samples did not differ between the groups. However, the levels of SOD were significantly ($p < 0.001$) higher in the CAPE-treated group (Tables 2 and 3).

Pancreatic tissue MDA decreased in the diabetic and CAPE groups at the end of the 60 days, compared to the control group. However, the levels of antioxidant enzymes (SOD and CAT) showed a non-significant downward trend (Table 3).

Hystopathological results

Normal histological structures were observed in tissues of the control group kidney (Fig. 1a), liver (Fig. 2a), and pancreas (Fig 3a). Similarly, CAPE group showed normal nephrotic findings compared to the diabetic group kidney tissue (Fig 1b). In this group, the pancreatic tissue (Fig. 3b) exhibited normal histological appearance similar to the control group, the liver hepatocytes showed some degenerative changes and vacuolization (Fig. 2c).

Hyperemia of the vessels, areas of focal congestion and macrophages in interstitium and mononuclear cell infiltration composed of lymphocytes and plasma cells were seen in kidneys of the diabetic group. (Fig. 1c). Hyaline cylinders in some of the tubule lumens, dilation and vacuolizations depended on glycogen degeneration and degenerative changes in tubules were observed (Fig. 1d). The increase in the mesangial matrix and shrinking of the bowman capsule were observed at some glomeruli. Hepatic cords had lost their shape. The sinusoidal regions

Table 3. Oxidant and antioxidant levels in kidney, liver and pancreas of control, CAPE and diabetic groups (mean±SEM)

	Control group (N:8)	Diabetic group (N:6)	CAPE group (N:8)	p
Kidney				
MDA (nmoL/g protein)	2.58±0.20[a]	2.33±0.22[a] (x0.90)	2.25±0.28[a] (x0.87)	0.585
CAT (k/g protein)	0.31±0.03[a]	0.36±0.05[a] (x1.16)	0.37±0.04[a] (x1.19)	0.435
SOD (U/mg protein)	0.25±0.01[b]	0.28±0.01[b] (x1.12)	0.33±0.01[a] (x1.32)	0.000
Liver				
MDA (nmoL/g protein)	1.69±0.07[b]	2.27±0.19[a] (x1.34)	1.78±0.10[b] (x1.05)	0.008
CAT (k/g protein)	0.40±0.04[a]	0.28±0.05[a] (x0.70)	0.43±0.05[a] (x1.08)	0.083
SOD (U/mg protein)	0.43±0.01[a]	0.40±0.04[a] (x0.93)	0.45±0.02[a] (x1.05)	0.466
Pancreas				
MDA (nmoL/g protein)	8.93±0.74[a]	3.96±0.72[b] (x0.44)	5.23±1.0[b] (0.59)	0.002
CAT (k/g protein)	0.043±0.008[a]	0.028±0.006[a] (x0.65)	0.038±0.008[a] (x0.88)	0.405
SOD (U/mg protein)	0.11±0.007[a]	0.106±0.007[a] (x0.96)	0.09±0.012[a] (0.82)	0.131

a, b, c; different letters in the same line are statistically significant (Tukey test, $p<0.05$). Parenthesis (x) explain xfold increase or decrease of parameters compared to control group.

were widened due to erythrocytes accumulation. Fat vacuoles occurred in the most of hepatocytes. In addition, the number of kupffer cells and degenerative changes in hepatocytes were activated (Fig. 2b). In pancreatic tissue, mononuclear cell infiltration consisting of macrophages and lymphocytes were seen in the interlobular septum (Fig. 3c). The vacuolizations depended on glycogen degeneration were observed in the islets of langerhans and the duct epithelium (Fig. 3d).

Discussion

Increased levels of reactive oxygen species (ROS) in DM are associated with reduction of antioxidant enzymatic activity (catalase, glutathione peroxidase-GSH-Px, superoxide dismutase-SOD) and non-enzymatic (vitamin A, C, and E, selenium, transferrin and lactoferrin) antioxidants [22]. In addition, the high glucose levels cause oxidative damage to tissue in the body [23,24].

Caffeic acid phenethyl ester (CAPE) is an active component of propolis. It exhibits antioxidant activity and effectively protects nuclear DNA, membrane lipids and cytosolic proteins against oxidative damage [13]. In this study, we investigated the effects of CAPE in STZ-induced diabetic rats. According to the results of this study, blood glucose level in diabetic group was significantly increased ($p<0.001$). In similar with control group, CAPE-treatment reduced also blood glucose levels (Table 1). Reduction of the glucose explain that CAPE may cause regeneration of beta cells, improve insulin sensivity and stimulate insulin secretion. A previous study supported this results [25]. Additionally, hystopathological findings in the CAPE group were observed almost normal and similar to the control group (Fig. 1b, 2c, 3b).

The level of blood glucose in control and CAPE groups was slightly higher than beginning of the study. Because the rats were put down by cervical dislocation under anesthesia and the anesthesia might increase the blood glucose. This finding was supported by Braslasu *et al*. [26] previously.

Oxidative stress is increased in experimental models of STZ-induced diabetes. STZ induces reactive oxygen species in pancreatic β-cells [6]. Thereby, β-cells do not produce enough insulin, causing hyperglycemia. Persistent hyperglycemia in DM causes an increase in production of ROS and disturb the balance between ROS production and cellular defense mechanisms. Consequently, all tissues are exposed glucose auto-oxidation and protein glycosylation. Tissue injury may cause cell dysfunction [27-29].

Kakkar *et al*. [30], reported that total and Cu-Zn SOD in diabetic pancreas continued to increase until 6 weeks.

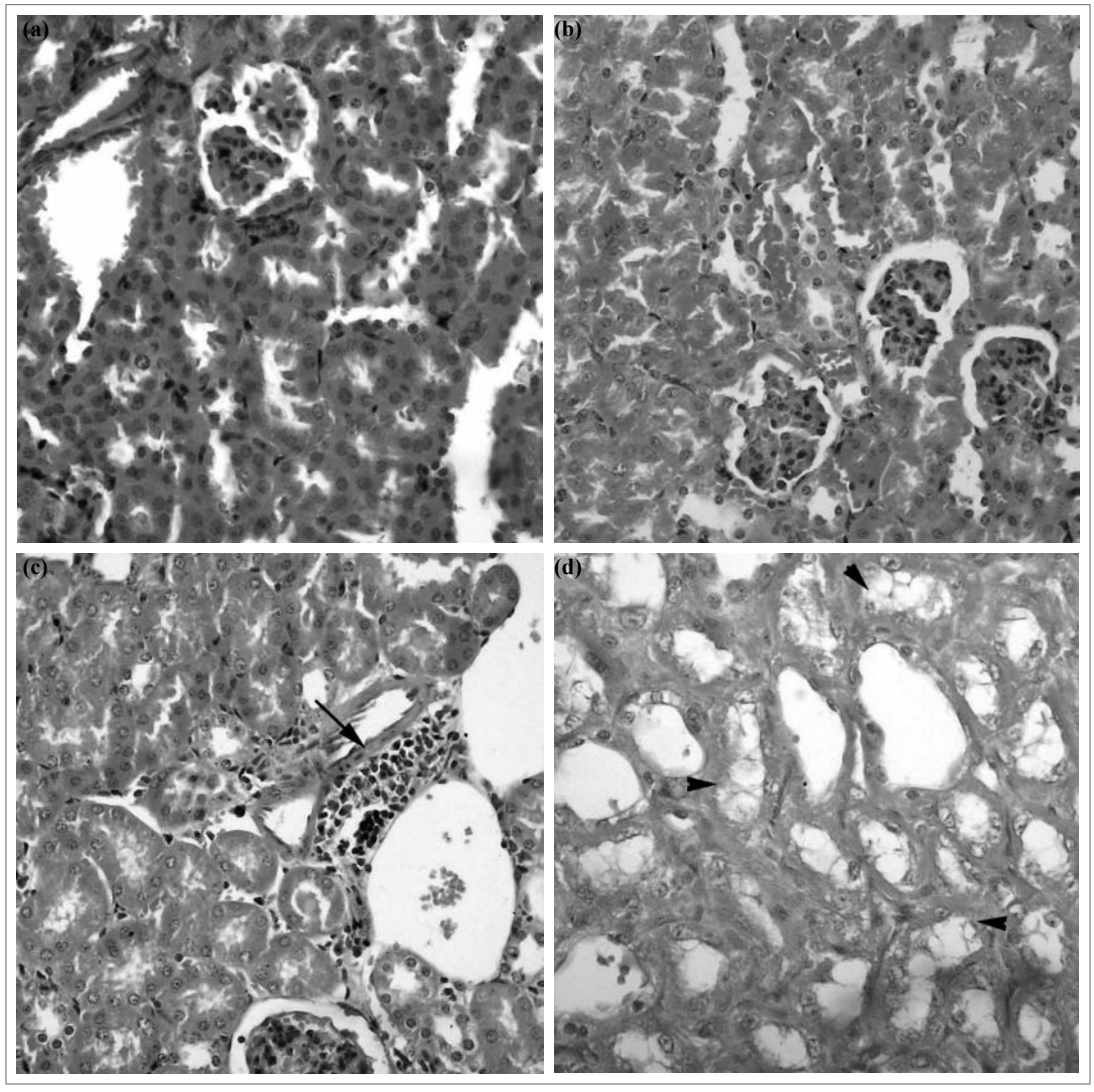

Figure 1. Normal histological structure of kidney in the control group **(a)**, CAPE group revealed near to normal histological structure **(b)** in diabetic group; interstitial inflammatory cell infiltration (arrow) and glycogenic vacuolization (arrow heads) with degenerative changes noted at tubules **(c, d)**, (H&Ex30).

After the sixth week, these enzymes decreased dramatically, but were still higher than controls. CAT and GSH-Px increased in the first period and then remained stable in diabetes. In the present study, pancreatic CAT tended to increase and SOD was almost unchanged in the CAPE group. Pancreatic MDA was decreased in the diabetes and CAPE groups (Table 3). This decreases might be exhausting MDA due to disruption in tissue integrity with STZ injection. Hystopathological examination of the pancreas in the diabetic group (Fig. 3c-d) indicated the mononuclear cell infiltration of interlobular septum and vacuolizations depended on glycogen degeneration in the ductal epithelium and islets of Langerhans, in similar to previous studies [31-33].

Bahram and Daryoush [34] determined that the antioxidant capacity significantly increased by the application of intraperitoneal aloe vera extract in STZ-induced DM. Moreover, lipid peroxidation, total SOD, Cu-Zn-SOD, GSH-Px and catalase activities in liver and pancreatic tissues increased in comparison with the control group. Mn-SOD activity remained unchanged. Our findings were similar at the end of 60 days and liver catalase and SOD activities in the CAPE group increased compared to diabetic group (Table 3).

The negative effect of DM on the liver is completely unknown [35,36]. The liver plays a significant role in maintaining glucose and fat metabolism [37]. It is reported that some degenerative changes in hepatocyte are lipolysis and vacuolation [31,33,38]. Our study observed, similar to other studies, lipid accumulation in liver due to degenerative changes and fat vacuoles in the cytoplasm of hepatocytes (Fig. 2b). The oxidative state supported pathological findings. MDA levels were significantly elevated in diabetic liver tissue and decreased in the CAPE group. Furthermore, ALT and AST levels elevated in the diabetic group (Table 2). Antioxidant enzymes tended to

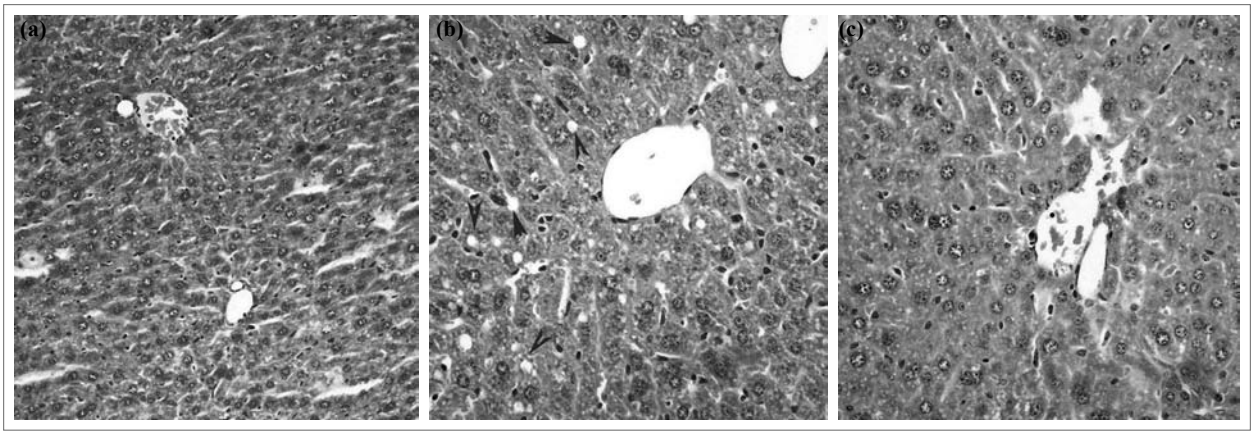

Figure 2. Normal histological structure of liver in the control group **(a)**, degenerative changes of hepatocytes and fat vacuoles (arrowheads) in diabetic group **(b)**, CAPE group; degenerative changes liver show near normal histological structure **(c)**, (H&Ex30).

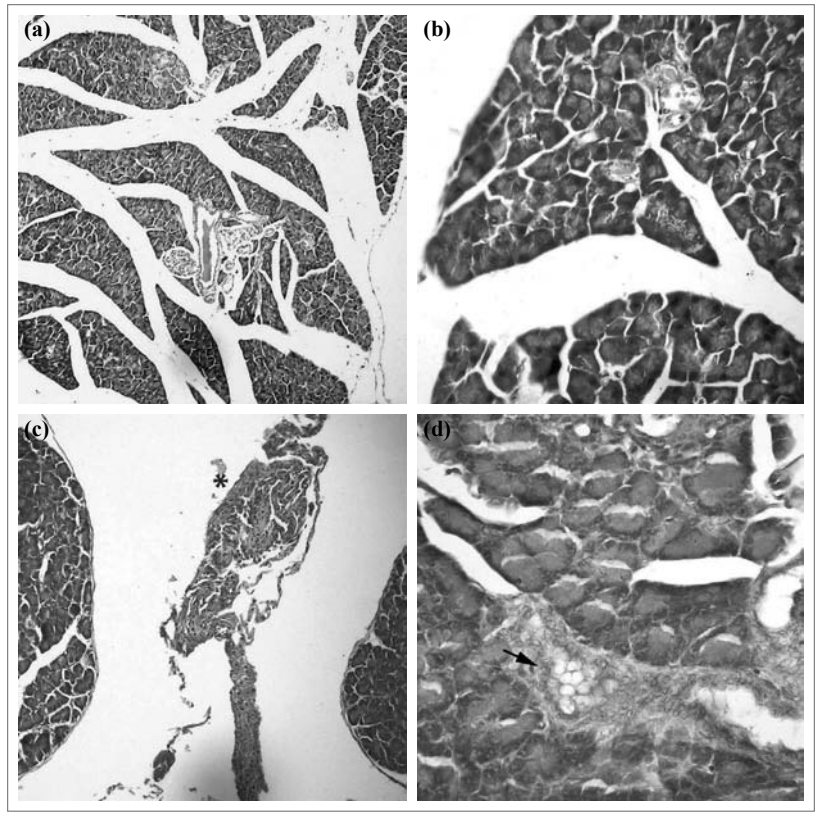

Figure 3. Normal pancreas tissue histology in the control group **(a)**, CAPE group pancreatic tissue exhibited normal histological appearance **(b)**, in diabetic group inflammatory cell infiltration observed in interlobular septum (*) and glycogenic vacuolization in the islets of langerhans (arrow) **(c,d)**, (a,b,c: H&Ex60, d: H&Ex30).

increase in the CAPE group (Table 3).

Hyperglycemia increases the production of oxygen free radicals and decreases antioxidant capacity. Oxidative stress affects the kidneys as other diabetic complications [39-41]. In our study, the levels of antioxidants in the kidneys of diabetic and CAPE groups tended to increase and so almost suppressed oxidative stress. This increase may be relatively associated with reducing MDA as the end product of lipid peroxidation. Renal SOD activity in CAPE group increased compared to the control and diabetic groups, and significantly ($p<0.001$) antioxidant effects of CAPE were observed (Table 3). In this context, the entrance of glucose into renal tissue was independent from insulin, so oxidative damage induced with hyperglisemia was considered to occur a longer period of time. These findings were supported by the normal range of creatinine. The slight increase in BUN is possibly related

to these physiopathological events (polyuria and polydipsia) in DM (Table 2).

Hystopathological examination of diabetic kidneys indicated interstitial nephritis, degenerative changes in tubules, vacuolizations depended on glycogen degeneration and glomerular nephropathy (Fig. 1c-d). These findings were similar to previous studies [31,38,42,43].

In conclusion, the antioxidant defense system deteriorated and decreased by oxidative injury in diabetes mellitus. Oxidative stress in diabetic liver might be due to lipid peroxidation. The kidney was affected a longer period. It was considered that the use of CAPE suppressed oxidative stress and decreased glucose levels in STZ-induced DM. Therefore, it may be useful to use as remedies, the antioxidant properties, in addition to anti-diabetic drugs in DM.

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Conflict of Interest

There are no conflicts of interest among the authors.

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