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MUSTAFA DURMUŞ

H. RAMAZAN YILMAZ

EFKAN UZ

NURTEN ÖZÇELİK

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## The Effect of Caffeic Acid Phenethyl Ester (CAPE) Treatment on Levels of MDA, NO and Antioxidant Enzyme Activities in Retinas of Streptozotocin-Induced Diabetic Rats

Mustafa DURMUŞ<sup>1</sup>  
H. Ramazan YILMAZ<sup>2</sup>  
Efkan UZ<sup>2</sup>  
Nurten ÖZÇELİK<sup>2</sup>

**Aim:** This study was performed to determine the effect of caffeic acid phenethyl ester (CAPE) treatment on levels of malondialdehyde (MDA) and nitric oxide (NO) and activities of antioxidant enzymes in retinas of streptozotocin (STZ)-induced diabetic rats.

**Materials and Methods:** Twenty-seven rats were enrolled and divided into three groups: group 1: non-diabetic rats as control (n = 9); group 2: STZ-induced, untreated diabetic rats (n = 8); and group 3: STZ-induced, CAPE-treated diabetic rats (n = 10). Levels of MDA and NO and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in retinal tissues were analyzed.

**Results:** In retinas of untreated diabetic rats, levels of MDA and NO and the activity of GSH-Px were significantly increased (P = 0.001, P = 0.0001, P = 0.0001, respectively), while the SOD activity was significantly decreased (P = 0.021) when compared to the control group. GSH-Px activity in the CAPE-treated diabetic group was markedly higher than that of the control group (P = 0.0001). MDA and NO levels were lower (P = 0.005, P = 0.001, respectively) and SOD activity was higher in the CAPE-treated diabetic group compared to the untreated diabetic group (P = 0.016).

**Conclusions:** It can be concluded that nitrosative and oxidative stress is increased in the diabetic retina. CAPE treatment inhibits lipid peroxidation and NO overproduction and regulates SOD enzyme activities in diabetic rat retinas while it has no effects on GSH-Px activity.

**Key Words:** Antioxidant enzymes, caffeic acid phenethyl ester, diabetic retinopathy, lipid peroxidation

### Streptozotocin ile Diyabet Oluşturulmuş Sıçanlarda Kafeik Asit Fenetil Ester (CAPE) Tedavisinin Retinadaki MDA, NO Düzeyleri ve Antioksidan Enzim Aktiviteleri Üzerine Etkileri

**Amaç:** Bu çalışma, streptozotocin (STZ) ile diyabet oluşturulmuş sıçanlarda, kafeik asit fenetil ester (CAPE) tedavisinin retinadaki malondialdehit (MDA), nitrik oksit (NO) düzeyleri ve antioksidan enzim aktiviteleri üzerine etkilerini araştırmak üzere yapıldı.

**Yöntem ve Gereç:** Yirmiyedi sıçan çalışmaya alındı ve üç gruba ayrıldı: grup 1: diyabetik olmayan sıçanlar (kontrol grubu) (N = 9); grup 2: STZ uygulanmış-tedavi uygulanmamış sıçanlar (N = 8); grup 3: STZ uygulanmış ve CAPE tedavisi verilmiş sıçanlar (N = 10). Bütün grupların retinalarında, MDA ve NO düzeyleri, superoksit dismutaz (SOD) ve glutatyon peroksidaz (GSH-Px) enzim aktiviteleri analiz edildi.

**Bulgular:** Kontrol grubu ile kıyaslandığında, tedavi uygulanmamış diyabetik sıçanlarda MDA ve NO düzeyleri ve GSH-Px aktivitesi artmış (sırasıyla P = 0.001, P = 0.0001, P = 0.0001), buna karşılık SOD aktivitesi önemli ölçüde azalmış olarak bulundu (P = 0.021). GSH-Px aktivitesi CAPE tedavisi uygulanmış diyabetik sıçanlarda, kontrol grubuna göre belirgin ölçüde yüksekti (P = 0.0001). CAPE tedavisi uygulanmış diyabetik sıçanlarda, tedavi edilmemiş olanlara nazaran MDA ve NO düzeyleri azalmış (sırasıyla P = 0.005, P = 0.001), SOD aktivitesi artmıştı (P = 0.016).

**Sonuç:** STZ ile diyabet oluşturulmuş sıçanlarda MDA ve NO düzeyleri ve GSH-Px aktivitesi artmıştı, CAPE tedavisi ile GSH-Px dışındakiler azalmaktaydı. SOD aktivitesi diyabetik grupta azalmışken, CAPE tedavisi ile artmaktaydı.

**Anahtar Sözcükler:** Antioksidan enzimler, kafeik asit fenetil ester, diyabetik retinopati, lipid peroksidasyonu

<sup>1</sup> Department of Ophthalmology,  
Faculty of Medicine,  
Fatih University,  
Ankara - TURKEY  
<sup>2</sup> Department of Medical Biology,  
Faculty of Medicine,  
Süleyman Demirel University,  
Isparta - TURKEY

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#### Correspondence

Mustafa DURMUŞ  
Department of Ophthalmology,  
Faculty of Medicine,  
Fatih University,  
Ankara - TURKEY

mudurmus@hotmail.com

## Introduction

Diabetic retinopathy (DR) is a major complication of diabetes and is a leading cause of blindness. DR has been considered a microvascular disease and the blood-retinal barrier breakdown is a hallmark of this disease (1). Diabetes mellitus is associated with an increased production of free radicals and consequent oxidative stress. Oxidative stress-related injury plays important roles in the development of diabetic complications (2,3). The majority of free radicals and toxic molecules are derived from oxygen. The principal free radicals are superoxide ( $O_2^{\bullet}$ ), hydroxyl ( $^{\bullet}OH$ ) and peroxy ( $LOO^{\bullet}$ ) radicals, all of which could play a role in DNA damage, glycation, and protein and lipid modification reactions in diabetes (4). Malondialdehyde (MDA) is an end product of lipid peroxidation and an indicator of lipid modification by free radicals in tissues containing high lipid components (i.e. the retina in our study). There is a basal release of nitric oxide (NO) that maintains the retinal blood flow in the retinal microcirculation (5). In diabetes mellitus, NO levels in tissues increase and the roles of increased NO in diabetes pathogenesis range from signal transmission to cell death (6). Antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) are parts of the protective defense mechanism against oxidative damage. These antioxidant defense mechanisms are also impaired in diabetes mellitus (7).

Caffeic acid phenethyl ester (CAPE), a flavonoid-like compound, is one of the major components of honeybee propolis. CAPE, which has no reported harmful effects on normal cells (8), has several biological and pharmacological properties: antioxidant (9), anti-inflammatory (10), anti-carcinogenic (11), antiviral (12), and immunomodulatory (13) activities. CAPE can completely block production of reactive oxygen species in human neutrophils and the xanthine/xanthine oxidase system at a concentration of  $10\mu M$  (9). The levels of lipid peroxidation and the activities of antioxidant enzymes were increased in streptozotocin (STZ)-induced diabetic rats (14,15). CAPE was shown to inhibit lipooxygenase activities and suppress lipid peroxidation (15).

We aimed to evaluate the protective effect of CAPE against oxidative stress in STZ-induced diabetic rat retinas in this study.

## Materials and Methods

### Animal Model

Twenty-seven male Sprague-Dawley rats (11 weeks old) were used in the study. They were kept in an environment of controlled temperature ( $24-26^{\circ}C$ ), humidity (55–60%), and controlled photoperiod (12 h light/dark cycle) during the experiment. A commercial balanced diet (Hasyem Ltd., Isparta, Turkey) and tap water were provided ad libitum. All animals received humane care, in compliance with the present institutional guidelines. The study was reviewed and approved by the Local Institutional Ethics Committee.

### Experimental Design

Twenty-seven rats were randomly divided into three groups (each animal placed separately in the stainless-steel cage) as follows: group 1, non-diabetic control rats ( $n = 9$ ); group 2, STZ-induced, untreated diabetic rats ( $n = 8$ ); and group 3, STZ-induced, CAPE-treated diabetic rats ( $n = 10$ ), which were injected daily with CAPE. CAPE was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and administered intraperitoneally (i.p.) at a dose of  $10\mu molL^{-1} kg^{-1} day^{-1}$ . CAPE was administered 3 days after the STZ treatment and continued until the time of sacrifice. STZ dissolved in sodium citrate buffer (pH 4.5) was administered i.p. at a single dose of  $35 mg kg^{-1}$  body weight (15). In control rats, isotonic saline solution (an equal volume of CAPE) was administered i.p. Blood glucose levels were measured with an Accu-Chek Active strip test in a glucometer (Roche Diagnostic, Mannheim, Germany) in all rats after 3 days of STZ treatment. The animals with blood glucose level less than  $300 mg dl^{-1}$  were excluded from the study. After eight weeks of CAPE treatment, the rats were anesthetized with an intramuscular (i.m.) injection of  $50 mg kg^{-1}$  ketamine hydrochloride (Ketalar, Eczacibasi, Istanbul, Turkey). All animals were sacrificed under anesthesia, their eyes enucleated and retinas carefully isolated. The specimens were harvested and stored at  $20^{\circ}C$  until biochemical assays.

### Biochemical Procedure

The frozen retinas were homogenized (Ultra Turrax T25, Germany) (1:10, w/v) in  $50 mmol L^{-1}$  phosphate buffer (pH 7.4) and kept in an ice bath. The homogenate was then centrifuged at  $5000g$  for 30 minutes to remove debris. The homogenate and supernatant were stored at  $20^{\circ}C$  in aliquots until used for biochemical assays. The protein content of the tissue was determined by using the Lowry method (16).

### Determination of MDA

Malondialdehyde (MDA) levels, an indicator of free radical generation that increases at the end of the lipid peroxidation, were estimated by the double heating method of Draper and Hadley (17). The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. The level of MDA is expressed as nanomoles per gram protein.

### Determination of NO Level

Nitric oxide (NO) measurement is very difficult in biological specimens because it can be readily oxidized to nitrite (NO<sub>2</sub>) and subsequently to nitrate (NO<sub>3</sub>), which serve as index parameters of NO production. The method for determining nitrite and nitrate levels was based on the Griess reaction (18). Results were expressed as micromoles per gram protein.

### Determination of SOD Activity

Total (Cu–Zn and Mn) SOD (E.C. 1.15.1.1) activity was determined according to the method of Sun et al. (19). The principle of the method is based, briefly, on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Activity was expressed as units per gram protein.

### Determination of GSH-Px Activity

Glutathione peroxidase (GSH-Px; E.C. 1.6.4.2) activity was measured by the method of Paglia and Valentine (20). The enzymatic reaction in the tube that contained reduced nicotinamide adenine dinucleotide phosphate, reduced glutathione, sodium azide and glutathione reductase was initiated by the addition of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was given in units per gram protein. All samples were assayed in duplicate.

### Statistical Analysis

Data were presented as means ± standard error (S.E.). The one-way analysis of variance (ANOVA) and post hoc multiple comparison tests (LSD) were performed on the data of biochemical variables to examine the difference among groups. P value of <0.05 was considered as statistically significant.

### Results

Initial and final body weight and final blood glucose levels of rats are shown in Table 1. Biochemical parameters are summarized in Table 2. MDA levels in the retina were increased in untreated diabetic rats when compared to those of the control and CAPE-treated diabetic groups (P = 0.001 and P = 0.005, respectively). In the CAPE-treated group, MDA levels were significantly lower when compared to untreated diabetic rats and were not significantly different from those of control

Table 1. Initial and final body weight and final blood glucose levels of rats in control, diabetic, and diabetic + CAPE-treated groups (Mean ± SE).

	Mean body weight (g) at the beginning of the study	Mean body weight (g) at the end of the study	Mean blood glucose levels (mg/dl) at the end of the study
1-Control (n = 9)	215 ± 10	296 ± 18	139 ± 09
2-Diabetic (n = 8)	216 ± 05	235 ± 07	385 ± 19
3-Diabetic + CAPE (n = 10)	219 ± 05	209 ± 08	384 ± 26
P-values			
1-2	n.s.	0.002	0.0001
1-3	n.s.	0.0001	0.0001
2-3	n.s.	n.s.	n.s.

n: number of rats; n.s.: not significant.

Table 2. Biochemical parameters of retinas in control, diabetic and CAPE-treated diabetic rats (Mean±SE).

	MDA (nmol/g protein)	NO (µmol/g protein)	SOD (U/mg protein)	GSH-Px (U/g protein)
1- Control (n=9)	22.73±4.58	1.62±0.28	0.14±0.01	0.76±0.02
2-Diabetic (n=8)	47.15±5.15	4.62±0.40	0.11±0.01	2.63±0.23
3-Diabetic+CAPE (n=10)	28.06±3.45	2.45±0.26	0.14±0.01	2.17±0.21
P values				
1-2	0.001	0.0001	0.021	0.0001
1-3	NS	NS	NS	0.0001
2-3	0.005	0.0001	0.016	NS

NS: not significant; n: number of rats; MDA: malondialdehyde; NO: nitric oxide; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; CAPE: caffeic acid phenethyl ester.

retinas. The levels of NO in retinas of untreated diabetic rats were higher than those of control and CAPE-treated diabetic groups ( $P = 0.0001$ ). NO levels in the CAPE-treated group were not significantly different from those of the control group.

Superoxide dismutase activities in the untreated diabetic group were significantly lower than in the control and CAPE-treated diabetic groups ( $P = 0.021$  and  $P = 0.016$ , respectively). There was no significant difference in SOD activities between the control and CAPE-treated diabetic rats.

On the other hand, the GSH-Px activities were significantly lower in the control group compared to both the untreated and CAPE-treated diabetic rats. The GSH-Px activities in the untreated diabetic group were not significantly different from those of the CAPE-treated group.

## Discussion

Diabetic complications have been linked to the increased production of free radicals in the tissues (3). Therefore, tissue antioxidant status is one of the major factors in determining the development of diabetic complications (21). The levels of MDA and NO and the activities of SOD and GSH-Px were determined in retinas of STZ-induced diabetic rats in this study.

Lipid peroxidation has been suggested to be closely related to diabetes-induced oxidative stress (2-4) and

MDA is a good indicator of the rate of lipid peroxidation (14,21). In the present study, CAPE treatment effectively prevented increase in MDA level in the diabetic rat retina ( $P = 0.001$ ) and hence retinal lipid peroxidation possibly through its antioxidant properties. We can say that CAPE treatment can prevent the lipid peroxidation in STZ-induced diabetic rat retinas.

Caffeic acid phenethyl ester possesses wide-ranging properties such as antioxidant, anti-inflammatory, anti-NF-kappaB, anti-cyclooxygenase and anti-lipoxygenase effects. The preventive effect of CAPE on lipid peroxidation in the retina can be explained by its oxidative stress-reducing effect and by its scavenging activity on superoxide radicals. CAPE markedly scavenges superoxide anions produced in both enzymatic and nonenzymatic reactions. In addition, it has been demonstrated that CAPE prevents the initiation of lipid peroxidation by scavenging peroxy radicals, and is known to be a more effective radical scavenger than trolox c, alpha-tocopherol, and ascorbic acid (22).

In the retinal microcirculation, there is a basal release of NO that maintains the retinal blood flow. NO can play either a neuroprotective or a neurotoxic role in diverse neurodegenerative conditions. It has been suggested that the pathogenesis of early DR may involve a reduced bioavailability or diminished production of NO [5]. On the other hand, hyperglycemia stimulates the production of advanced glycosylated end products, activates protein kinase C, and enhances the polyol pathway leading to increased superoxide anion formation. Superoxide anion

interacts with NO, forming the potent cytotoxin peroxynitrite, which attacks various biomolecules in the vascular endothelium, vascular smooth muscle and myocardium, leading to cardiovascular dysfunction. The pathogenetic role of nitrosative stress and peroxynitrite and downstream mechanisms including poly (ADP-ribose) polymerase (PARP) activation contribute to the development and progression of diabetic nephropathy, retinopathy and neuropathy (23). In our study, NO levels were markedly increased in the diabetic rat retinas and this increase was inhibited by CAPE treatment, bringing NO values to those of the control group. This result demonstrated that CAPE has an ability to reduce the nitrosative stress in the diabetic rat retina.

Antioxidant enzymes protect tissues against oxidative damage. For example, SOD catalyzes the conversion of superoxide radical to H<sub>2</sub>O<sub>2</sub> and protects the cells against toxic effects of superoxide radicals. GSH-Px eliminates H<sub>2</sub>O<sub>2</sub> and lipid hydroperoxides and reduces peroxides by using reduced glutathione as a hydrogen donor (24). In the current study, SOD activity was significantly decreased in untreated diabetic rat retinas. CAPE treatment enhanced the SOD activity in diabetic rats. Our result is consistent with the result of Obrosova (25), who indicated a decrease in SOD activity in the diabetic rat retina. Reduced antioxidant levels as a result of increased free radical production in experimental diabetes have

been shown in several studies (7,25). The retinal GSH-Px activity was significantly increased in untreated diabetic and CAPE-treated diabetic rats compared to the controls. The increased GSH-Px activity in diabetic rats reflects the increased production of H<sub>2</sub>O<sub>2</sub>, in accordance with the findings of other studies (14,15,26). In the present study, CAPE treatment did not change the activity of GSH-Px in the diabetic retina.

It can be concluded that nitrosative and oxidative stress is increased in the diabetic retina. CAPE treatment inhibits lipid peroxidation and NO overproduction and regulates SOD enzyme activities in diabetic rat retinas, while it has no effects on GSH-Px activity. The protective role of CAPE might be related with its antioxidant properties. The results imply that CAPE can be beneficial to prevent or decrease oxidative and nitrosative stress in the diabetic retina. However, further studies are needed to prove the protective effect of CAPE on oxidative stress in the retina caused by diabetes mellitus.

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