

Effects of Caffeic Acid Phenethyl Ester on Retinal Damage After Traumatic Brain Injury

Efectos del Ester Fenético del Ácido Cafeico en el Daño de la Retina Después de Una Lesion Cerebral Traumática

Turgut Fethiye Gülden¹; Özevren Hüseyin²; Yıldırım M.¹ & Deveci, E.³

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SUMMARY: Head trauma damages the optic nerve visual function and visual acuity. Effects of head trauma on the retina was investigated with biochemical, histological and immunohistochemical respects. The study was conducted on 30 rats with three groups: group 1 was control group (n=10). Second group was head-traumatized group (n=10) and last group was head-traumatized+Caffeic acid phenethyl ester (CAPE, i.p. 20ml/kg/day). Upon head was traumatized, CAPE was applied to trauma+CAPE group and then for the following four days. At the end of 5th day, rats were anesthetized with ketamine hydroxide and then blood samples were taken for biochemical analysis. MDA and GSH-Px values were compared. After blood sample, total eyes of rats were dissected for histopathological and immunohistochemical analysis. In trauma group, degeneration in retinal photoreceptor cells, disintegrity and in inner and outer nuclear layers, hypertrophy in ganglion cells, and hemorrhage in blood vessels were observed. In the group treated with CAPE, lesser degeneration in photoreceptor cells, regular appearances of inner and outer nuclear layers, mild hemorrhage in blood vessels of ganglionic cell layer were observed. The apoptotic changes caused by trauma seen in photoreceptor and ganglionic cells were decreased and cellular organization was preserved due to CAPE treatment. CAPE was thought to induce healing process on traumatic damages.

KEY WORDS: Traumatic Brain injury; Retina; Caffeic acid phenethyl ester (CAPE); Rat.

INTRODUCTION

Severity of traumatic brain injury is commonly described as mild, moderate, or severe based on a number of factors such as the duration of loss of consciousness and/or coma rating scale or score, posttraumatic amnesia, and brain imaging results (Saatman *et al.*, 2008). Concussion, or mild TBI (mTBI), is the most common form of TBI, accounting for up to 75 % of all brain injuries occurring annually in the United States (Faul *et al.*, 2010).

Traumatic optic neuropathy (TON) occurs in 0.5-5 % of patients in the USA presenting with closed head trauma (Steinsapir & Goldberg, 1994). Recently, changes in surrogate measures of visual function and ocular structures have been reported in rodent studies using a single blast

injury focused on the head. The authors found a decrease in retinal nerve fiber layer (RNFL) thickness and a decrease in pattern electroretinography (ERG) 3 to 4 months after injury, which correlated with punctate regions of reduced cellularity in the ganglion cell layer and damage to the optic nerve (Mohan *et al.*, 2013). VEGF is known to play a crucial role in the pathogenesis of diabetic macular edema by promoting blood-retinal barrier (BRB) disruption and exacerbated vascular leakage (Zhang *et al.*, 2008). A previous study has shown that systemic neutralization of VEGF in mice resulted in substantial neural retinal cell death (Saint-Geniez *et al.*, 2008). Damage to the optic nerve (ON) causes immediate shearing of and induces secondary swelling in a proportion of retinal ganglion cell

¹ Departments of Ophthalmology, Gazi Yasargil Education and Research Hospital Diyarbakir, Turkey.

² Departments of Neurosurgery, Faculty of Medicine, University of Dicle, Diyarbakir, Turkey.

³ Departments of Histology and Embryology, Faculty of Medicine, University of Dicle, Diyarbakir, Turkey.

(RGC) axons, accompanied by subsequent RGC degeneration (Sarkies, 2004). GFAP is a 51-kDa intermediate filament protein found in the astrocytes, Müller glial cell end-feet and processes. Although Müller glial cells in normal rat retinas express little or no GFAP (Bignami & Dahl, 1979), they showed increased GFAP expression in retinal injuries including ischemia (Larsen & Osborne, 1996), glaucoma (Xue *et al.*, 2006) and kainate-induced neuronal death (Honjo *et al.*, 2000). Some of the investigators suggest a pivotal role of MMP inhibition in glaucoma treatment. Inhibition of MMP-9 could inhibit the apoptosis of retinal ganglion cells and tissue remodeling (Mozaffarieh & Flammer, 2007).

A member of the matrix metalloproteinases (MMPs), a family of zinc-dependent proteases that can degrade components of the ECM, is a potential candidate for this purpose MMP expression is associated with tumor invasion and metastatic potential (Beliën *et al.*, 1999).

Kinouchi *et al.* (2003) recently demonstrated that elimination of the intermediate filaments of reactive Müller cells or astrocytes within the retina permitted neuronal integration in retinal transplantation of glial fibrillary acidic protein (GFAP), vimentin mice. Caffeic acid phenethyl ester (CAPE) is one of the major components of honeybee propolis and has been used in traditional medicine. It was found to be a potent free radical scavenger and antioxidant (Ilhan *et al.*, 1999). In this study, the protective effects of CAPE were investigated on changes occurred in eye retinal layer by trauma with immunohistochemical, histopathological and biochemical methods.

MATERIAL AND METHOD

The investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication no. 85-23, revised 1996). All experimental protocols were approved by the Dicle University Animal Care and Use Committee. Male Sprague-Dawley rats (300–350 g) were housed in an air-conditioned room with 12-h light and dark cycles, where the temperature (23 ± 2 °C) and relative humidity (65–70 %) were kept constant. The animals were anesthetized by an intraperitoneal injection of 5 mg/kg xylazine HCl (Rompun, Bayer HealthCare AG, Germany) and 40 mg/kg ketamine HCl (Ketalar, Pfizer Inc., USA), and were allowed to breathe spontaneously. A rectal probe was inserted, and the animals were positioned on a heating pad that maintained the body temperature at 37 °C. The widely used diffuse brain injury model

described by Marmarou *et al.* (1994) was used. Briefly, a trauma device which works by dropping a constant weight from a specific height through a tube was used. A weight of 300 g was dropped from a 1 m height, which can induce mild trauma. In this study, 30 rats were randomly divided into three groups each with 10 rats: group 1 was control group. Second group was head-traumatized group and third group was head-traumatized+ CAPE. CAPE was intraperitoneally administered with dose of 20 ml/kg/day. After head trauma, CAPE was applied to trauma+CAPE group (also can be named CAPE group) for five days. At the end of 5th day, rats were anesthetized with ketamine hydroxide and then blood samples were taken for biochemical analysis. MDA and GSH-Px values were compared. Later, whole eyes of rats were dissected for histopathological and immunohistochemical analysis. Retina of eyes was extracted and was fixed in a 10 % formalin solution and embedded in paraffin blocks for histopathologic examination. 5 µm thick sections were obtained from paraffin blocks and stained with Hematoxylin- Eosin(H-E).

Malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) assays. The MDA level and GSH-Px activity were determined in each retina and the average values of each group were calculated. The retina samples were prepared as a 10 % homogenate in 0.9 % saline using a homogenizer on ice according to weight. Then, the homogenate was sedimented at 2000 rpm for 10 min, and the supernatant was collected and diluted. The MDA levels were determined using the double heating method of Draper & Hadley (1990). MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. Briefly, 2.5 ml of TBA solution (100 g/L) were added to 0.5 ml of homogenate in each centrifuge tube, and then the tubes were placed in boiling water for 15 min. After cooling with flowing water, the tubes were centrifuged at 1000 rpm for 10 min, 2 ml of the supernatant were added to 1 ml of TBA solution (6.7 g/L), and the tube was placed in boiling water for another 15 min. After cooling, the amount of thiobarbituric acid-reactive species (TBARS) was measured at 532 nm. The MDA concentration was calculated from the absorbance coefficient of the MDA–TBA complex. Data are expressed as nanomoles per gram (nM/g) wet tissue. The GSH-Px activity was measured by the method of Paglia & Valentine (1967). 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 H₂O₂, and the change in absorbance at 340 nm was monitored by spectrophotometry. Data were expressed as U/g protein.

Immunohistochemical staining. An antigen-retrieval

process was performed in citrate buffer solution (pH 6.0) two times: first for 7 min, and then for 5 min in a microwave oven at 700 W. They were allowed to cool down to room temperature for 30 min and washed in distilled water for 5 min twice. Endogenous peroxidase activity was blocked in 0.1 % hydrogen peroxide for 15 min. Ultra V block (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 10 min prior to the application of the primary antibodies (VEGF antibody, mouse monoclonal, 1/200, Santa Cruz Biotechnology, GFAP antibody, mouse monoclonal, 1/200, Santa Cruz Biotechnology and MMP9 antibody, mouse monoclonal, 1/100, Santa Cruz Biotechnology) overnight. The secondary antibody (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 20 min. Then the slides were exposed to streptavidin-peroxidase for 20 min. Diaminobenzidine (DAB, Invitrogen, Carlsbad) was used as a chromogen. Control slides were prepared as mentioned above but omitting the primary antibodies. After counterstaining with Hematoxylin, washing in tap water for 5 min, and in distilled water for 2 × 5 min, the slides were mounted.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism 4.0 software (GraphPad Software, 2003, San Diego, CA, USA). All data are presented as mean ± standard deviation (SD). Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's (Table I).

RESULTS

Tissue MDA levels. The MDA values of the tissues were compared between the groups. The trauma group had higher MDA value than control and trauma+CAPE group. MDA level was low in CAPE treated group ($p < 0.001$). There was no significant difference between the CAPE+trauma group and the control group ($p = 0.314$).

Tissue glutathione peroxidase (GSH-Px) levels. GSH-Px levels in trauma group was lower than levels in control and CAPE group (0.001). CAPE group had higher GSH-Px level than trauma group did (0.001). There was no significant difference between the CAPE group and the control group ($p < 0.05$).

Histopathologic Analysis. In the retinal sections of control groups, normal appearance of the internal and outer nuclear layers and the normal organization of the retinal ganglion cell layer are seen.

CAPE treatment. CAPE was thought to induce healing process on traumatic damages (Fig.1).

Immunohistochemical Analysis. Weak VEGF expression was observed in the outer limiting membrane and pigment layer of control group sections. VEGF was positively expressed in inner limiting membrane and ganglion cells. Blood vessels around ganglion layer had positive VEGF expression in trauma group. Trauma+CAPE group had less VEGF expression than trauma group had.

In the control group, GFAP protein expression was observed in the ganglion cell layer and inner and outer layers plexiform. In the trauma group, an increase of GFAP protein expression in the ganglion cell layer and inner and outer layers plexiform was observed (Fig. 2).

GFAP was positively expressed in ganglionic cells of trauma+CAPE group. Positive MMP9 expression was observed in retinal pigment epithelium layer, inner and outer limiting membrane and ganglionic layer. Ganglionic cells of trauma group had positive MMP9 expression. While trauma+CAPE group had positive MMP9 expression in inner and outer limiting membrane and in plexiform layer, MMP9 expression was weakly in ganglionic layer.

Table I. Statistical analysis

	Control	CAPE	Trauma	Trauma+ CAPE
MDA (nM/g)	21.30±1.54	20.41±1.37	34.33±1.36***	26.66±1.58+++
GSH-Px (U/g)	24.35±0.57	22.24±1.14*	15.69±0.76***	19.85±1.17+++

Values are represented as mean ± SD. ; *** $p < 0.001$, versus control; * $p < 0.05$, versus control; +++ $p < 0.001$, trauma+ CAPE versus trauma

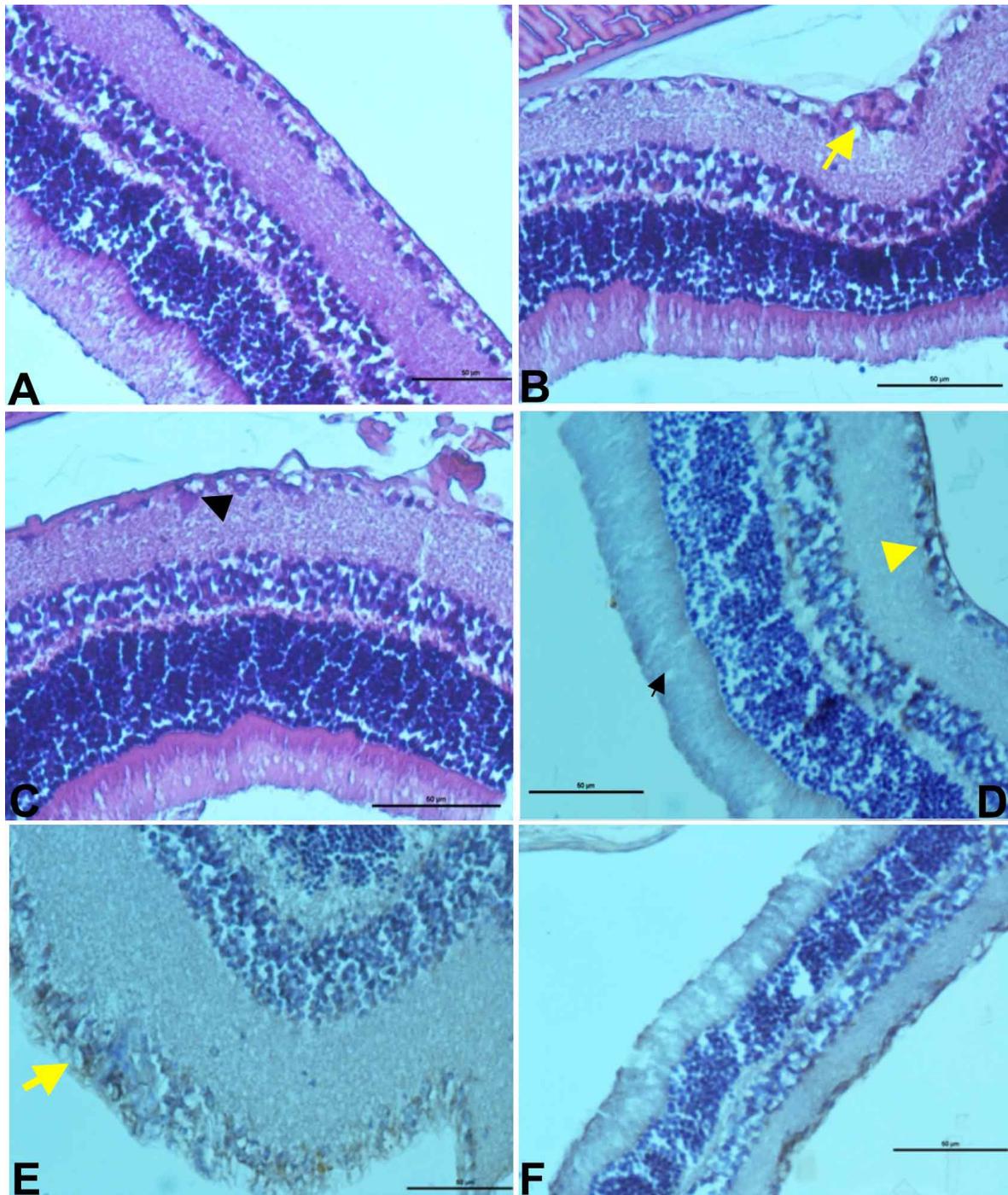


Fig. 1a-Control group: Normal appearance of the internal and outer nuclear layers and the normal organization of the retinal ganglion cell layer, H-E Staining Bar 50 µm. b-Trauma group: Some degenerative changes in retinal layers and hypertrophy in retinal ganglion cells are seen. Also, separations of the cells are observed both in internal nuclear layer and outer nuclear layer, H-E Staining Bar 50 µm. c-Trauma+Capegroup: Integrity of the inner and outer nuclear layer, disorganization in some ganglion cells(arrow), H-E Staining Bar 50 µm. d-Control group: Weak VEGF expression in the outer limiting membrane and retinal pigment epithelium(arrow), Positive VEGF expression in the inner limiting membrane, ganglion cell layer (yellow arrow), VEGF immunohistochemical staining Bar 50 µm. e-Trauma group: An increase of VEGF expression in the ganglion layer. VEGF immunohistochemical staining Bar 50 µm. f-Trauma+Capegroup: VEGF positive expression in a thin layer(arrow), VEGF immunohistochemical staining Bar 50 µm.

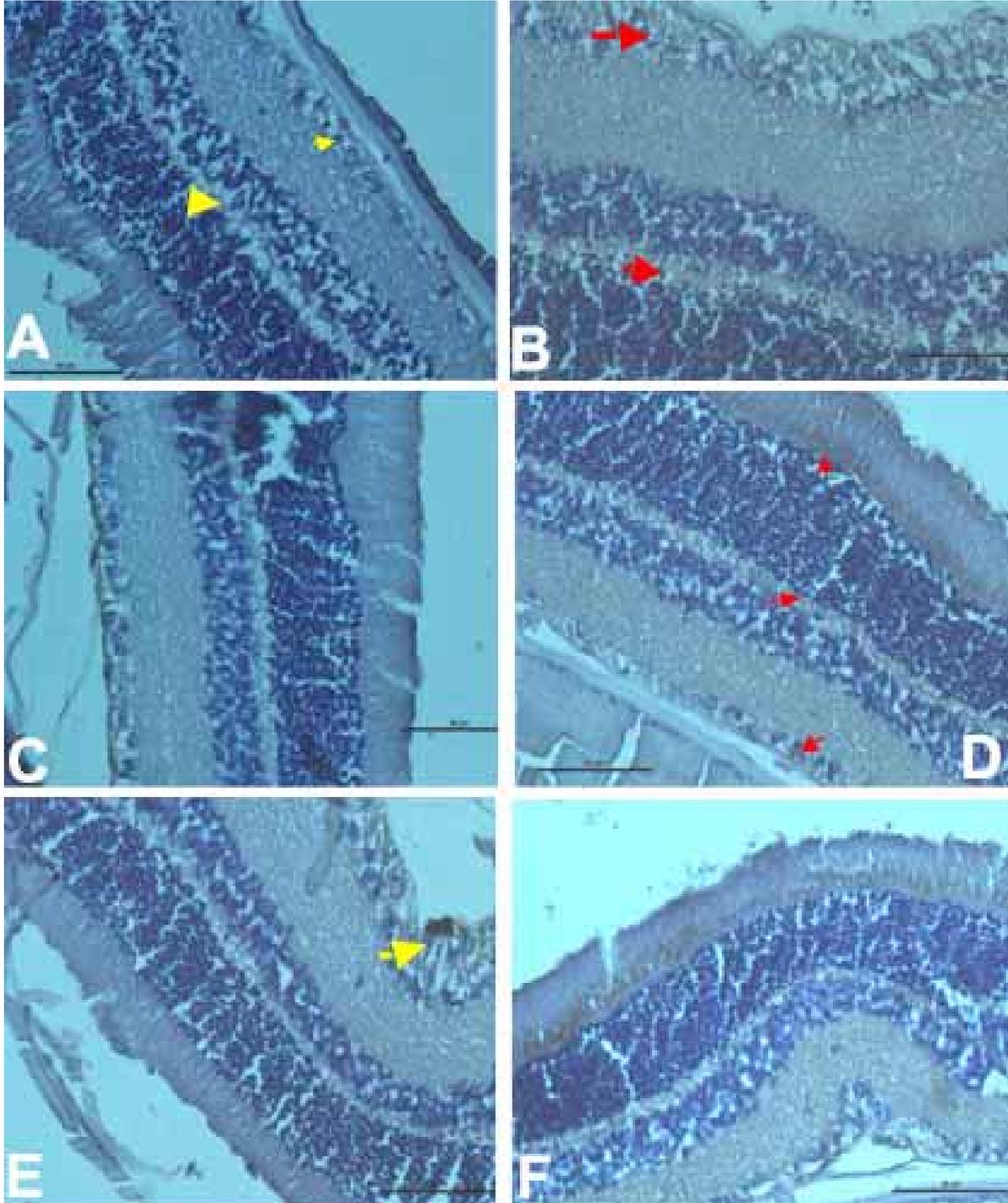


Fig. 2a-Control group: GFAP protein expression in the ganglion cell layer and inner and outer layers plexiform (yellow arrow), GFAP immunohistochemical staining Bar 50 μ m. b-Trauma group: An increase GFAP protein expression in the ganglion cell layer and inner and outer layers plexiform, (red arrow) GFAP immunohistochemical staining Bar 50 μ m. c-Trauma+Capegroup:Positive GFAP expression was observed regularly in the ganglion cell layer,GFAP immunohistochemical staining Bar 50 μ m.d- Control group: MMP-9 expression in the inner layer of the retina ganglion cells and in the lower area of the pigment layer(red arrow) MMP9 immunohistochemical staining Bar 50 μ m. e-Trauma group:An increase MMP9 expression in ganglion cell layer MMP9 immunohistochemical staining Bar 50 μ m. f-Trauma+Cape group MMP-9 expression in the inner layer and in the lower area of the pigment layer(arrow)also weak MMP9 expression in ganglion cell layer.

DISCUSSION

Depending on the level of traumatic brain injury, symptoms such as visual complaints, blurredness, loss of visual acuity, loss of visual acuity and visual field defects may occur (Brahm *et al.*, 2009). Pathologic findings in the optic nerve after moderate to severe TBI have been well documented and include ischemic necrosis and shearing lesions most often localized near the optic foramen (Crompton, 1970). Early degeneration of the endothelium by free radical damage, production of advanced glycation end-products (AGE) and hypersecretion of various basement membrane proteins may affect transport, permeability and integrity of tight junctions and the blood-retinal barrier. A low oxygen tension stimulates vascular endothelial growth factor (VEGF) synthesis and VEGF receptor expression in an attempt to revascularize the ischemic retina (Cagliero *et al.*, 1991). In our study, while VEGF was weakly expressed in retinal pigment epithelium and outer limiting membrane, it was positively expressed in inner limiting membrane and ganglionic cells. Increased VEGF expression was clearly observed in ganglionic cells of trauma group. VEGF expression was decreased in blood vessels of ganglion layer in CAPE group. MMP-9 is associated with inflammatory cell migration and extracellular matrix degradation, and the pro-form of MMP-9 is significantly elevated in the neovascular retinal membranes (Das *et al.*, 1999a). Increased MMP-9 is also observed in retinas with active neovascularization (Das *et al.*, 1999b). Robertson *et al.* (2013) observed that MMP-9 plays an important role in maintaining intraocular pressure, and due to extracellular matrix remodeling, leads to ocular hypertension and glaucoma.

According to a study, after traumatic injury, inner layer of retina, ganglionic cell layer and optic nerves showed no MMP-9 expression (Zalewska *et al.*, 2016). This study showed that in trauma-treated group, increased MMP9 expression has been observed in the ganglion layer. Chintala *et al.* observed that MMP-9 deficiency protects against pathological changes in the retina after optic nerve ligation. They found that increased MMP-9 activity is associated with laminin degradation, which results in retinal ganglion cell apoptosis (Chintala *et al.*, 2002). GFAP is a 50-52 kDa acidic cytoskeletal protein. It is a marker protein of colloid cell, with plenty and exclusive expression in it. GFAP can be used to mark the post trauma changes of colloid cell, and normally GFAP is under the dynamic adjustment of glia cells (Zhang *et al.*, 2004). Post trauma proliferated GFAP-positive colloid cell can promote caryocinesis of colloid cell, causing the differentiation of primitive progenitor cell towards mature glia cell. Various kinds of central nervous system lesion can

induce colloid cell response (Cao *et al.*, 2001). Apoptotic effect in photoreceptor cells observed after traumatic injury, changes in limiting membranes, degeneration in ganglionic cells, and additionally hemorrhage in blood vessels influences visual function. It was seen that post-traumatic CAPE treatment reduced apoptotic effect, preserved membrane continuity, supported regulation of neuronal association in ganglionic cells. We thought that CAPE treatment may induce apoptotic and angiogenic effects.

TURGUT, F. G.; ÖZEVREN, H.; YILDIRIM, M. & DEVECI, E. Efectos del ester fenetílico del ácido cafeico en el daño de la retina después de una lesión cerebral traumática. *Int. J. Morphol.*, 36(1):97-103, 2018..

RESUMEN: El trauma craneal daña la función visual del nervio óptico y la agudeza visual. Se investigaron los efectos del traumatismo craneal en la retina con aspectos bioquímicos, histológicos e inmunohistoquímicos. El estudio se realizó en 30 ratas distribuidas en tres grupos: grupo control (n = 10); grupo con traumatismo craneal (n = 10); grupo con traumatismo craneoencefálico + Éster fenetílico de ácido cafeico (CAPE, i.p. 20 ml / kg / día). Sobre la cabeza traumatizada, se aplicó CAPE a trauma + grupo CAPE durante los siguientes cuatro días. Al final del día 5, las ratas se anestesiaron con hidróxido de ketamina y luego se tomaron muestras de sangre para el análisis bioquímico. Se compararon los valores de MDA y GSH-Px. Después de la muestra de sangre, se disecaron los ojos de las ratas para su análisis histopatológico e inmunohistoquímico. En el grupo de traumatismos, se observó degeneración en las células fotorreceptoras retinianas, desintegridad en capas nucleares internas y externas, hipertrofia en células ganglionares y hemorragia en los vasos sanguíneos. En el grupo tratado con CAPE, se observó una menor degeneración en las células fotorreceptoras, apariciones regulares de capas nucleares internas y externas, hemorragia leve en los vasos sanguíneos de la capa de células ganglionares. Los cambios apoptóticos causados por el trauma visto en el fotorreceptor y las células ganglionares disminuyeron y la organización celular se conservó debido al tratamiento con CAPE. Se concluyó que CAPE induce un proceso de curación en daños traumáticos.

PALABRAS CLAVE: Lesión cerebral traumática; Retina; Éster fenetílico del ácido cafeico (CAPE); Rata.

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Corresponding author:

Dr. Engin Deveci

Department of Histology and Embryology

Dicle University Faculty of Medicine

Diyarbakır

TURKEY

Email: engindeveci64@gmail.com

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