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Original Article

Caffeic acid phenethyl ester (CAPE) prevents formaldehyde-induced neuronal damage in hippocampus of rats

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ABSTRACT

The aim of this study was to investigate the neurotoxicity of formaldehyde on hippocampus and the protective effects of caffeic acid phenethyl ester (CAPE) against these toxic effects. For this purpose, 21 male Wistar rats were divided into three groups. The rats in Group I comprised the controls, while the rats in Group II were injected every day with formaldehyde (FA). The rats in Group III received CAPE daily while exposed to formaldehyde. At the end of 8 days experimental period, all rats were sacrificed by decapitation. The brains of the rats were removed and the hippocampus tissues were obtained from all brain specimens. Some of the hippocampus tissue specimens were used for determination of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels. The remaining hippocampus tissue specimens were used for light microscopic and immunohistochemical evaluation.

The levels of SOD, GSH-Px and MDA were significantly increased in rats treated with formaldehyde compared with those of the controls. Furthermore, in the microscopic examination of this group, formation of apoptotic bodies, pycnotic cells, and vacuoler degeneration areas were observed. However, decreased biochemical parameters were detected in the rats administered CAPE while exposed to formaldehyde. Additionally, cellular damage caused by formaldehyde was decreased, and structural appearance was similar to that of the control rats in this group. These biochemical and histological findings observed in all groups were also confirmed by the immunohistochemical evaluation.

It was determined that formaldehyde-induced neuronal damage in hippocampus was prevented by administration of CAPE. ***Neuroanatomy; 2007; 6: 66–71.***

Key words [caffeic acid phenethyl ester] [formaldehyde] [hippocampus] [neurotoxicity] [rat]

Introduction

The hippocampus is a bilaterally symmetrical structure shaped somewhat like a cashew nut and extends along the ventromedial border of the temporal lobe. Hippocampus is a cytoarhitecturally distinct structure folded into the cerebral cortex. It has been shown to be involved in the integration of information arriving from different sensory organs and associated areas and is essential for memory storage and retrieval, playing an important role in declarative memory. The hippocampus is the most critical brain area for our ability to recollect everyday events and factual knowledge. This ability is what in everyday language is referred to as 'memory', although it should more precisely be called declarative memory, as opposed to other types, such as procedural memory, which is unconscious and hippocampus-independent recollection of information. The function of hippocampus is regulated by cholinergic innervations that arise mainly from the medial septum. Lesioning the medial septum removes the hippocampal cholinergic innervation and it induces a memory defect in experimental animals [1,2]. In humans, hippocampectomy disrupted the memory formation in almost all kinds of tasks that require new learning, in particular declarative memory, leaving only implicit forms of long-term memory, such as procedural memory and priming intact [3,4]. In rats, however, some tasks were relatively unaffected by the damage of the hippocampus, for example recognition memory [5] and fear conditioning [6]. The most dramatic memory failure in animals was observed in tasks that required spatial memory [7]. In the light of this, any damage of the hippocampus may cause disorders in the functions of the hippocampus.

Formaldehyde (FA), a member of aldehyde family and one of the simplest organic molecules, is a pungent, irritant and colourless gas. It is found in nature in foods, domestic air, cigarette smoke, and the polluted atmosphere of cities due to the incomplete combustion of organics, photochemical smog and release from FA containing products. Thus, everyone living in society may be exposed to it. FA is also widely used in industrial and medical areas and employees may be highly exposed to it in these settings. Especially, anatomists and medical students having dissection lectures are the most common subjects that can be exposed to FA gas [8-10]. Formaldehyde is accepted as toxic over certain doses and the chances of harmful effects are increased under room temperature because of its volatility [8,9,11,12]. Formaldehyde (FA), an occupational and environmental toxicant used extensively in the manufacturing of many household, personal use products. In hospitals, it is used for pathology work, hemodialysis and in medical technology units [13].

It is known that inhaled FA gas has negative effects on the central nervous system, and these effects may appear acutely in the form of headaches, malaise, sleeping disorders, fatigue, anorexia and dizziness [14]. Long-term exposure to FA may cause irreversible neurotoxicity [15] and is related to central nervous system cancer (brain astrocytoma) [16]. In addition, inhaled FA has been shown

to cause behavioral and memory disorders in rats and has been classified as 'probably neurotoxic' [17].

Caffeic acid phenethyl ester (CAPE) is an active component in honeybee propolis extracts and is considered to have medicinal properties. It has anti-inflammatory, immunomodulatory, anti-proliferative and anti-oxidant properties and has been shown to inhibit both lipooxygenase activity and suppress lipid peroxidation [18-23]. However, to our best knowledge, there is no experimental study concerning the protective effects of CAPE against neuronal damage in the hippocampus induced by FA.

Therefore, the present study aimed to examine the neurotoxic effects of formaldehyde on hippocampus and to investigate the protective effects of CAPE against these toxic effects at biochemical, light microscopic and immunohistochemical levels.

Materials and Methods

Animals and Treatments

Adult male Wistar rats (weighing 200–250 g) obtained from Firat University Medical Faculty Experimental Research Unit were randomly divided into three groups with seven animals per group. All animals received humane care in compliance with the European Community Guidelines on the care and use of laboratory animals (86/609/EEC). The rats were kept in plexiglas cages (4 rats/cage) where they received standard chow (supplied from Elazig Feed Plant, Elazig, Turkey) and water ad libitum in an air-conditioned room with automatically regulated temperature (21 ± 1 ºC) and lighting (07.00-19.00 h).

The animals were divided into three groups. The rats in Group I (n = 7) were used as the controls. The rats in Group II (n = 7) were injected with formaldehyde (FA) (10 mg/kg, intraperitoneally) daily. The rats in Group III (n = 7) received CAPE (10 μg/kg, intraperitoneally) daily while exposed to FA. At the end of the eight days experimental period, all rats were sacrificed by decapitation. The brains of rats were removed and hippocampus tissues were obtained from all the brain specimens. Some of the hippocampus tissue specimens were washed twice with cold saline solution, placed into glass bottles, labeled, and stored frozen (-30ºC) for eventual determination of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) production. The other hippocampus tissue specimens were used for light microscopic and immunohistochemical evaluations.

Biochemical analysis of prefrontal cortex tissues

For biochemical analysis, the tissues were weighed and homogenized in four volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 0.50 ml/l Triton X-100 with a homogenizer (IKA Ultra-Turrax T 25 Basic) for 2 min at 13.000 rpm. All procedures were performed at +4°C. Tissue MDA levels were determined in the homogenate. Tissue homogenates were then centrifuged at 5000 g for 60 min to remove debris. Clear supernatant fluids were separated and kept at -40°C until the enzyme activity measurements were performed (about a week).

Determination of superoxide dismutase activity

Total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was determined based on the method of Sun et al [24]. The principle of the method is based on the inhibition of Nitro Blue 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 ml of 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 amount of enzyme causing 50% inhibition in the NBT reduction rate. The SOD activity was expressed as U/g protein.

Determination of glutathione peroxidase activity

Glutathione peroxidase (GSH-Px, EC 1.6.4.2) activity was measured by the method of Paglia and Valentine [25]. The enzyme reaction in the tube containing NADPH, reduced glutathione (GSH), sodium azide and glutathione reductase was initiated by addition of H_2O_2, and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was expressed as U/g protein.

Determination of malondialdehyde level

The tissue malondialdehyde (MDA) level was determined using a method by Esterbauer and Cheeseman [26] based on reaction with thiobarbituric acid (TBA) at 90-100°C. In the TBA test reaction, MDA and TBA react to produce a pink pigment with an absorption maximum at 532 nm. The reaction was performed at pH 2-3 and 90°C for 15 min. The sample was mixed with two volumes of cold 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was centrifuged and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water-bath for 10 min. After cooling, the absorbance was read at 532 nm. Results were expressed as nmol/g wet tissue, by reference to a standard curve prepared from measurements made with a standard solution (1,1,3,3-tetramethoxypropane).

Microscopic examination of hippocampus tissue specimens

The hippocampus tissue specimens were fixed in neutral formalin solution (10%). Tissue specimens were embedded in paraffin wax and sectioned (thickness, 5 μm). Paraffin sections were used for light microscopic and immunohistochemical examination. For light microscopic evaluation, paraffin sections were stained with hematoxylin-eosin (HE) and examined with an Olympus BH2 light microscope.

Immunohistochemical procedure

For immunohistochemical Bax staining (a marker protein of apoptosis), paraffin sections were deparaffinized in xylene, hydrated and then placed in phosphate buffered saline (PBS; pH 7.6). Antigen retrieval was performed by boiling for 15 min in citrate buffer (0.01 M). Sections were treated with 3% hydrogen peroxide for 5 min to quench endogenous peroxidase activity, rinsed with deionized water and then washed with PBS. Sections were incubated first with 1% pre-immune rabbit serum to decrease non-specific staining and then with a monoclonal antibody against Bax protein (Dako, Carpinteria CA, USA) at 23 °C in a moist chamber for 1 hour. Detection of the antibody was performed using

Table 1. MDA, GSH-Px and SOD values in the hippocampus of the three groups of rats (n = 7 for each group).

Group	MDA (nmol/g)	GSH-Px (U/g)	SOD (U/g)
I (Control)	20.3 ± 0.6	54.2 ± 2.1	192.1 ± 5.1
II (FA)	31.4 ± 0.9	74.3 ± 3.3	262.4 ± 5.6
III (FA+ CAPE)	24.0 ± 0.7	57.2 ± 2.2	221.6 ± 3.9
p< (I vs II)	0.001	0.001	0.001
p< (I vs III)	0.001	n.s.	0.001
p< (II vs III)	0.001	0.001	0.001

Values are expressed as means ± SD. n.s., non significant; FA, formaldehyde; CAPE, caffeic acid phenethyl ester)

a biotin-streptavidin detection system (Bio-Genex, San Ramon CA, USA) with 3-amino 9-ethyl carbazole (AEC) as chromogen (Dako, Carpinteria CA, USA). Sections were counterstained with Mayer's hematoxylin, dehydrated and then cover-slipped with Permount.

Immunoreactivity for the Bax protein was scored semi-quantitatively. Stained sections were evaluated using a light microscope with the results expressed as a score based on the percentage of the total field stained positively with monoclonal antibody against Bax protein. Scores were based on the following scale; (heavy, 5+): over 80% of the field showing positive staining; (strong, 4+): 60%-79%; (moderate 3+): 40%-59%; (low 2+): 20%-39%; (minimal, 1+): 1%-19%; and no staining (0).

Statistical analysis

Quantitative data (SOD, GSH-Px and MDA values) are expressed as means ± standard deviations (SD). All statistical analyses were made with the statistical software package SPSS, version 11.0 (SPSS, Chicago, IL, USA). Data were tested for normality using the Kolmogorov-Smirnov test. Since all data were found to be normally distributed, within group comparisons were made using one-way ANOVA followed by LSD post-hoc test. The level of significance was set at $p< 0.05$.

Results

Clinical findings

The clinical evaluation of the rats which were systemically injected with formaldehyde revealed that there were no reductions in their water and food consumption but they had faded hairs and slow motor activity. Besides these symptoms the frequencies of grooming was increased. However the rats that were received CAPE along exposed to FA showed fewer symptoms and behavioral disorder than those observed in the FA administered rats.

Biochemical findings

SOD and GSH-Px values of FA-administered rats were significantly higher than the SOD and GSH-Px values of the control group ($p<0.001$). In addition, the MDA level, which is considered to be an important parameter for the determination of oxidative damage, was significantly higher in the FA-administered group than in the control group ($p<0.001$). The rats that were exposed to FA together with CAPE administration had decreased SOD, GSH-Px and MDA levels compared to FA-administered rats ($p<0.001$; Table 1).

Light microscopic findings

In the light microscopic evaluation, the hippocampus tissue sections of the control group that were stained with HE had a normal appearance. The nuclei of the neurons had normal sizes and regular membranes (Fig. 1). On the other hand, the histological appearance of the hippocampus tissue sections obtained from FA administered rats revealed apoptotic cells with fragmented nucleus. Furthermore, shrinking pycnotic cells were at the point, apoptotic bodies and vacuolar degeneration areas (Fig. 2). In the rats exposed to FA along with CAPE treatment, the cellular damage due to FA administration was decreased and their neuronal structure was similar to that of the control group (Fig. 3).

Immunohistochemical findings

The hippocampus sections obtained from every group were immunohistochemically stained with Bax and the results were semi-quantitatively evaluated. Bax-stained cells were not observed in the control group (0), (Fig. 4), while FA administered rats had dense immune positive cells (4+) (Fig. 5). The group that was exposed to FA along with CAPE administration had minimal immune positive cells (1+) (Fig. 6).

Discussion

Formaldehyde (FA), which leads to irritation of mucous membranes, has also been reported to have toxic effects on the respiratory tract (the lungs, nose), gastrointestinal system, skin, eye, testicles and menstrual functions [27-29]. FA has also a strong tendency to combine with protein, DNA, RNA which leads to allergic reactions, cytotoxicity, genotoxicity, mutagenic and cancerogenic actions [8-10].

The central nervous system is one of the most important systems affected by the effects of FA. Anatomists, histology, pathology, cadaver embalming technicians, dissection students, and nurses working at dialysis units are occupationally subject to FA exposure. There have been reports of malaise, headache, indigestion, balance and sleep disorders, and mental and memory disorders due to the exposure [13,28,30]. Moreover, the reports of severe fatigue and feeling of thirst, irritability, lethargy, and behavioral and sensory-emotional disorders in the people working in the industrial areas where FA is regularly used are suggestive of neurotoxicity [15,30].

The neurotoxic effects of FA were shown in experimental studies. It was reported that acute FA administrations at low concentrations increases the stimulation rate of the central nervous system, while applied in higher concentrations, it acts as a depressant on the central nervous system [10]. The studies on rats reported that exposure to FA to slows down motor activity [10,31]. In the study of Pitten et al [17], FA inhaling rats had inhibited learning capacity. Furthermore, other studies on rats have also reported that exposure of FA causes behavioral sensitivity and hesitation [32].

Similarly in our study, the rats that were systemically exposed to FA had several symptoms such as marked slow motor activity. Thus, the clinical findings of these rats were parallel to the findings of the previous studies mentioned above [10,17,31,32]. At the same time we detected increased frequencies of grooming in FA group

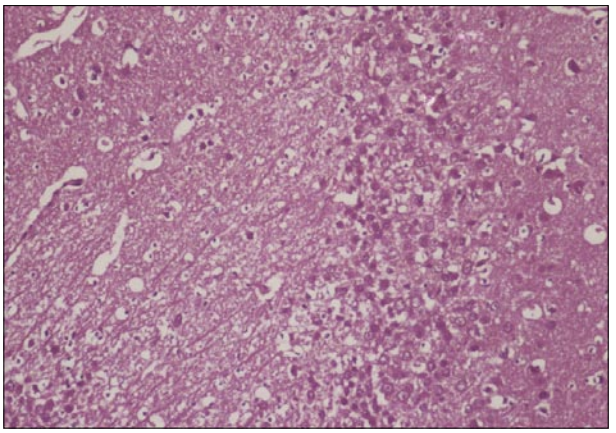

Figure 1. Hippocampus section from control group showing a normal histological appearance. Color version of figure is available online. (H.E. Magnification, X20)

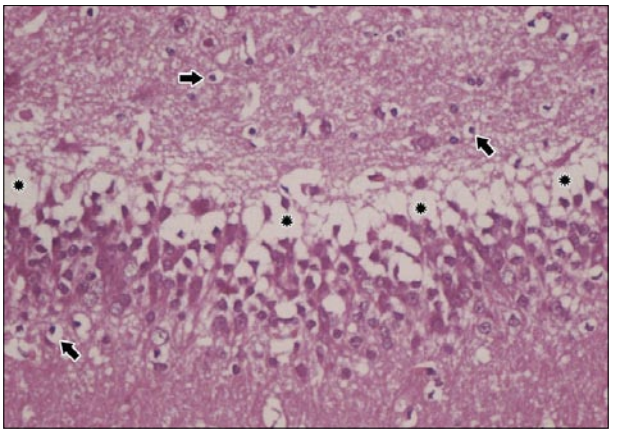

Figure 2. Pycnotic cells (***arrows***) and vacuolar degeneration areas (***asterisks***) were observed in hippocampus of FA administered rats. Color version of figure is available online. (H.E. Magnification, X20)

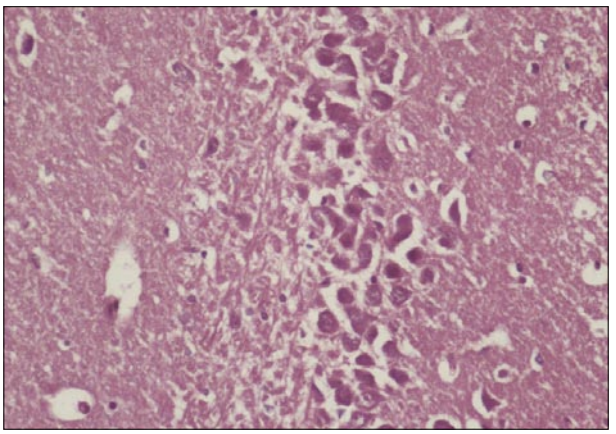

Figure 3. Hippocampus section of rats treated with FA and CAPE. The cellular damage observed after administration of FA was decreased and the neuronal structure was similar to that of the control group. Color version of figure is available online. (H.E. Magnification, X20)

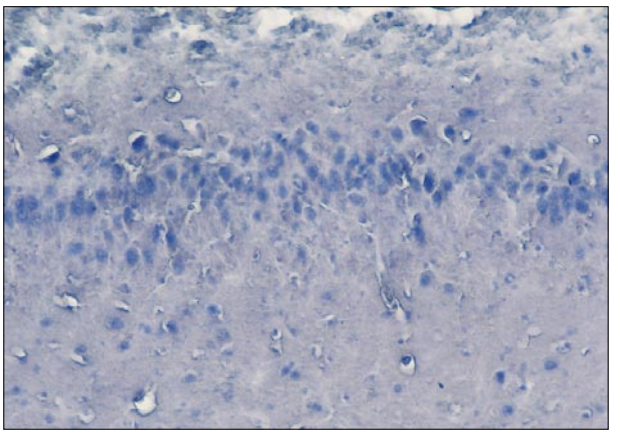

Figure 4. Immunohistochemically, bax-stained cells were not observed in hippocampus of control rats. Color version of figure is available online. (Magnification, X20)

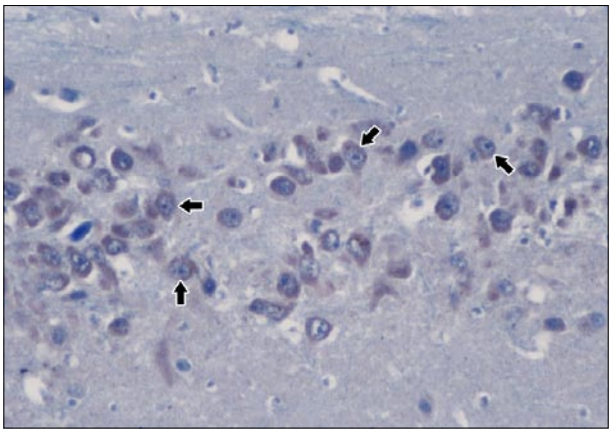

Figure 5. The percentage of bax immune positive cells (***arrows***) was high in rats treated with FA. Color version of figure is available online. (Magnification, X40)

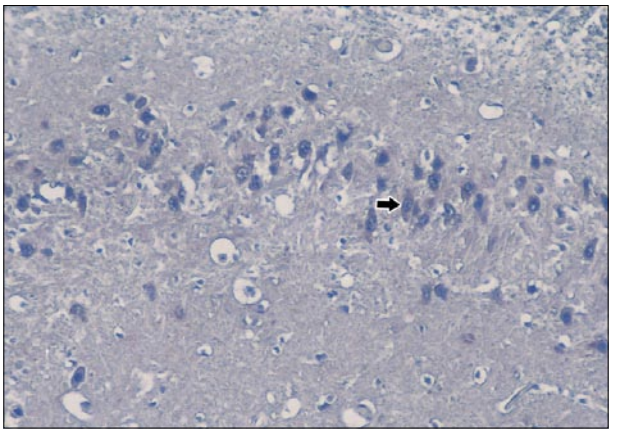

Figure 6. TThe density of immunohistochemically bax-stained cells (***arrow***) was minimal in rats treated with FA and CAPE. Color version of figure is available online. (Magnification, X20)

rats. We evaluated this symptom as a hippocampal neuronal damage so increasing this symptom mentioned as behavioral change by Malek et al [33].

The balance between the free radicals formed under normal physiological conditions or because of a pathological event and the antioxidant defense system, which protects them, may tend to shift in favor of free radicals, which indicates oxidative stress. Living organisms are protected against oxidative damage by enzymatic or non-enzymatic antioxidant system and molecules. Superoxide dismutase (SOD), glutation peroxidase (GSH-Px) and catalase (CAT) are among the enzymatic antioxidant systems effective at cellular level. The increased antioxidant enzymes enhance the antioxidant potential of the organs to reduce oxidative stress. There is considerable evidence that exposure to an oxidative stress can induce antioxidant enzymes, such as catalase and Mn-superoxide dismutase (SOD), in a variety of systems and that increased expression of Mn-SOD can protect against oxidant injury [34]. In our study, the hippocampus tissue that was exposed to FA had significantly increased SOD and GSH-Px activities, which indicates that FA had disrupted the antioxidant defense mechanism of hippocampus, causing oxidative damage. Similarly, Teng et al [32] in their experimental study on isolated rat hepatocytes found that FA, even at low concentrations, causes oxidative damage.

MDA is one of the products formed after lipid peroxidation and is a commonly used parameter for the determination of oxidative damage [35]. In our study, the MDA levels of FA administered group were significantly higher than those of the controls. This showed that FA had caused lipid peroxidation of the hippocampus; thus, leading to oxidative damage. Teng et al [32] reported similar results after FA administration in their study on rats.

Apoptotic process is histologically characterized by shrinkage of cells, increased chromatin density, nuclear picnosis and destruction, stoplasmic budding, and apoptotic body formation. The cells with damaged DNA exterminate themselves to prevent the damage to the organism. To achieve tissue homeosteasis, cells vanish in the medium by dying [36]. Neuronal cell death is observed in conditions and diseases such as brain trauma, spinal cord injury, Alzheimer, Parkinson and Huntington [37]. DNA damage is an intracellular signal for apoptosis [38]. FA causes apoptosis by inflicting DNA damage [39].

It has been reported that the cells of central nervous system may become apoptotic due to damage by various external factors such as toxins, trauma, radiation [40]. In the study as reported here, the tissue sections of the FA administered group had apoptotic cells with broken nuclei. There were also pycnotic cells and apoptotic body formations. Likewise, in previous studies, FA has been reported to cause DNA damage, thereby, leading to apoptosis [41-43].

Mitochondria play an important role in the apoptotic process. Death signals cause an increase in the permeability of the outer mitochondrial membrane, which in turn causes apoptosis. Some proteins regulate the permeability of the outer membrane of mitochondria. The most important of them are the proteins of Bcl-2 family. Some of the proteins in this family are pro-apoptotic, whereas some are anti-apoptotic. Bax is a pro-apoptotic protein and it causes cytochrome c release into cytoplasm from the mitochondrial membrane. Then, cytchrome-c starts the apoptotic process by activating caspase in the cytoplasm. However, Bcl-2 is a anti-apoptotic protein and it inhibits cytochrome c release by preventing Bax insertion into the mitochondrial membrane. To sum up, immunohistochemical Bax staining in cytoplasm of the cells indicates apoptosis has occurred [44,45]. In the present study, we showed that FA caused apotosis in the hippocampus.

CAPE is an active component of honeybee propolis extracts and it has been shown to be a pharmacologically-safe compound with anti-inflammatory, anti-mitogenic, anti-carcinogenic, anti-oxidant and immunomodulatory effects [18-22]. Previous experimental studies have shown that lipid peroxidation can be prevented by CAPE in spinal cord and kidney after ischemia-reperfusion [46,47]. Ozen et al [48] have shown that CAPE administration prevents cisplatin-induced nephrotoxicity in rats. Similarly, Fadillioglu et al [23] have reported that CAPE inhibits cardiotoxicity induced by doxorubicin in rat. Additionally, it has been reported that CAPE has protective effects against oxidative damage in various tissues [49-51]. CAPE has these protective effects on the basis of anti-oxidant actions, but the exact mechanisms of anti-oxidant properties of CAPE are not known yet. However, it has been speculated that CAPE may affect transcription and/or translation of genes and gene products of anti-oxidant enzymes [48].

In the present study, it has been shown that FA-induced neurotoxicity is prevented by CAPE. In other words, the SOD, GSH-Px and MDA levels of the rats exposed to FA along with CAPE had decreased. Furthermore, light microscopic examination of this group showed that the apoptotic changes due to FA exposure had disappeared and the neuronal structures were more like those of the control group. Additionally, the density of immunohistochemically Bax-stained cells was minimal in this group.

In conclusion, the findings obtained in the present study indicate that FA causes neuronal damage in the hippocampus of rats, which can be prevented by CAPE.

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