Beneficial Effects of Caffeic Acid Phenethyl Ester on Formaldehyde-Induced Learning and Memory Disabilities: A Labyrinth Test Performance Study

Formaldehitin Neden Olduğu Öğrenme ve Hafıza Yetmezlikleri Üzerine Kafeik Asit Fenetil Esterin Yararlı Etkileri: Bir Labirent Testi Performans Çalışması

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Abstract
Purpose: In this study, it was aimed to investigate the efficacy of caffeic acid phenethyl ester (CAPE) on formaldehyde (HCHO)-induced learning and memory changes in rats.
Material and Methods: For this purpose, labyrinth test was performed on 21 male Wistar rats. These animals were divided into three equal groups. All groups underwent training period in the labyrinth test. At the end of this period, control rats were injected intraperitoneally with ethanol; the rats in HCHO group were injected intraperitoneally with formaldehyde, and the rats in HCHO+CAPE group were injected with CAPE along with formaldehyde. All animals, within injection period, were subjected to labyrinth test again. The time spent by each rat in reaching the target was recorded and statistically evaluated using one-way variance analysis (ANOVA) and two-way ANOVA for repeated measures.
Results: Formaldehyde was observed to cause defects in learning and memory, and changes in behavior in rats. The time used by the HCHO-exposed rats in reaching the target was longer than that of control rats. This negative effect of HCHO exposure was partially alleviated by CAPE administration.
Conclusion: In view of the present findings, it is suggested that caffeic acid phenethyl ester exerts beneficial effects against formaldehyde toxicity.

Key Words: **Caffeic acid phenethyl ester; Formaldehyde; Neurotoxicity; Rat.**

Özet
Amaç: Bu çalışmanın amacı, formaldehitin (HCHO) sıçanlarda neden olduğu öğrenme ve hafıza değişiklikleri üzerine kafeik asit fenetil esterin (CAPE) etkinliğinin araştırılmasıdır.
Gereç ve Yöntemler: Bunun için, Wistar cinsi 21 erkek sıçana labirent testi uygulandı. Hayvanlar üç eşit gruba bölündü. Bütün gruplar labirent testinde eğitim sürecinden geçti. Bu süreç sonunda, kontrol sıçanlara intraperitoneal (ip) etanol, HCHO grubundaki sıçanlara ip formaldehit ve HCHO+CAPE grubundaki sıçanlara ise formaldehit ile birlikte CAPE enjeksiyonları yapıldı. Enjeksiyon dönemindeki tüm hayvanlara yeniden labirent testi uygulandı. Her bir sıçanın hedefe varma süresi kaydedildi ve tek yönlü varyans analizi (ANOVA) ile tekrarlı ölçümler için çift yönlü ANOVA kullanılarak istatistiksel olarak değerlendirildi.
Bulgular: Formaldehitin sıçanlarda öğrenme ve hafıza bozuklukları ile davranış değişikliklerine yol açtığı gözlendi. HCHO'ya maruz kalan sıçanların hedefe ulaşmada kullandıkları süre kontrol sıçanlarınkine oranla daha uzundu. HCHO maruziyetinin bu olumsuz etkisi CAPE uygulamasıyla kısmen giderildi.
Sonuç: Bu bulgular ışığında, kafeik asit fenetil esterin formaldehit toksisitesine karşı yararlı etkiler gösterdiği söylenebilir.

Anahtar Kelimeler: **Kafeik asit fenetil ester; Formaldehit; Nörotoksisite; Sıçan.**

Submitted : January 18, 2008
Revised : February 20, 2008
Accepted : July 21, 2008

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Introduction

Formaldehyde (HCHO) is an extensively used substance as an embalming solution in the areas of anatomy, histology and pathology. With its sharp odor, it is a colorless and irritating member of aldehyde family (1,2). Used in paints, plastic, construction materials, paper, cosmetic products, and cigarette smoke, it is commonly found in the environment (3,4). In addition, it is benefited from in hemodialysis equipment. Despite such variety of use, HCHO poses many potentially detrimental effects to body systems (5). In the studies performed on the experimental animals, HCHO has been found to harm respiratory system (6). Long-term HCHO inspiration at a dose of 15 ppm causes squamous cell carcinomas in the nasal cavities of rats and mice (7). The workers at a carpet factory who were occupationally exposed to HCHO presented loss of respiratory functions (8). Furthermore, while oral exposure to HCHO led to gastrointestinal irritation in rats, those whose drinking water was HCHO added had increased rates of thickening of stomach mucosa, atrophic gastritis, focal ulceration, hyperplasia, and hyperkeratosis (9). HCHO has also carcinogenic effects. The industrial workers who were exposed to HCHO had increased incidence of mouth and pharynx cancers (10). The toxic and carcinogenic effects of HCHO have been attributed to its binding capacity with DNA and proteins (11).

The neurotoxic effects of HCHO on the central nervous system have also been shown (2,4,12). Malek et al. (13) claimed that even one dose of HCHO leads to behavioral changes. The epidemiological studies performed on humans have determined that long-term exposure to HCHO in the work place leads to neurovegetative complaints such as headache, fatigue, indigestion, and nausea. Additionally, it has been held responsible for sleep disorders, memory disorders, mental state changes, and increased irritability (14,15).

Caffeic acid phenethyl ester (CAPE), an active component of the propolis, is a substance with a sharp and pleasant odor that is found in the plant extracts accumulated by bees (16). It has been used as a folk medicine for many years (17). CAPE is known to have antioxidant, immunomodulatory, anticarcinogenic and anti-inflammatory effects (18). It has been shown to produce a neuroprotective effect by blocking the production of reactive oxygen species (19). Furthermore, some investigators have shown the cytotoxic effect of CAPE on tumor cells and the cells transformed by virus, but no effects on the normal cells (20).

This study, performed with the labyrinth test on male rats, aimed to investigate whether caffeic acid phenethyl ester treatment prevents formaldehyde-induced neurotoxicity. For this purpose, we have examined influences of exposure to formaldehyde on the learning behavior of adult male rats and possible protective effects of caffeic acid phenethyl ester on behavioral regression of formaldehyde treated-rats.

Materials and Methods

Preparation of CAPE. Caffeic acid phenethyl ester (CAPE) applied in this study was synthesized in the Physico-Chemistry Laboratory according to standard method described by Grunberger et al. (21).

Animals. Adult male Wistar albino 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) in an air-conditioned room with automatically regulated temperature (22±1ºC) and lighting (07.00 to19.00 h). They received water ad libitum and were fed twice a day with standard chow (supplied from Elazig Feed Plant, Elazig, Turkey), with a food restriction regimen before and during labyrinth test periods. To prevent the rats being affected by various smells, the medium they were kept in was regularly aired and assured to be odorless. The wood shavings at the bottom of the cages were replaced twice a day to avoid smells. In order to accustom them with the smell, pieces of cheese were added to the pellet food consumed by the rats.

Test Procedure. A labyrinth mechanism used by Pitten et al. (22) was defined as the testing apparatus. In our modified mechanism, a labyrinth with a size of 100x100x20 cm was constructed in a way to have open top, divided compartments with wooden walls, and corridors. Some openings were left in three of the compartments for rats to enable easy passage. The raw material used for labyrinth construction was formica covered chipboard. The starting and target points were identified on the labyrinth (Figure 1). For rats to find the target, a small piece of cheese was placed. The time spent in finding the cheese was recorded and the data were statistically evaluated.

Figure 1. The "start (small arrow) and end (large arrow)" points in the labyrinth setting used in the experiment.

Training Period. The rats were divided into three equal groups: The controls, HCHO and HCHO+CAPE groups. The rats were released to the labyrinth at the starting point once a day for five days for familiarization. At the end of the familiarization period, a piece of cheese was placed into the target compartment for ten days and the rats found the cheese with the help of an observer. In the following ten days, the time they needed to find the cheese was recorded. In this process, the numbered animals were placed individually in the labyrinth and allowed time for locating the cheese. Before each rat was released into the labyrinth, any stool and urine that released odor was cleaned off the labyrinth with the help of a wet sponge. Therefore, any possible odor production was prevented. After the medium was completely dry, the next animal was admitted to the test process.

Treatments. All injections were performed intraperitoneally following the training period. The control rats received ethanol (10 μmol/kg/day) for eight days, while rats in HCHO group injected with HCHO (10 mg/kg/day) starting from the 4^{th} day of the experiment. In HCHO+CAPE group, rats were administered CAPE (10 μmol/kg/day) starting from the first day throughout eight days and HCHO (10 mg/kg/day) starting from the 4^{th} day. All animals received the determined agents at determined doses for eight days; however, on the 9^{th} and the 10^{th} days, none of them were injected. The time spent by the animals during this period was also recorded. Additionally, some behavioural parameters were registered manually by an independent observer, who was blind to the exposure regimen.

Statistical Analyses. Quantitative data were expressed as mean ± standard deviation. Distributions within the groups were analysed with one-sample Kolmogorov-Smirnov test. All three groups showed normal distribution, so that parametric statistical methods were used to analyze the data. For statistical significance, one-way variance analysis (ANOVA) and two-way ANOVA for repeated measures were used, where appropriate. The differences were compared by Tukey test with post-hoc group comparisons. All the statistical analyses were carried out with SPSS 11.0 for windows software (SPSS Inc., Chicago, IL, USA) and differences were considered significant at $P<0.05$.

Results

Clinical Findings. Even it is not very marked, the hairs of the rats in the HCHO group were pale, and they exhibited slowed-down locomotor activity compared to control group. On the other hand, the animals in this group displayed more grooming acts than usual (licking themselves or using their paws, performing self-cleaning). Nevertheless, the body weight and food and water consumption did not reduce. The rats that were exposed to HCHO along with CAPE, however, showed fewer symptoms and behavioral disorders than those observed in the HCHO group rats.

Labyrinth Test Findings. No difference was detected among the groups with respect to the time used to locate the food in the target before the injections (Figure 2 and Table I). During injection period, the rats in HCHO group used longer time to find the food in the labyrinth test than did the rats in the control group. The time used to find the food by the rats exposed to HCHO+CAPE was shorter than the time used by the rats in the HCHO group, almost about the time used by the control group rats (Figure 3 and Table II). The comparisons of the time recorded for each group showed that the duration of time used by HCHO group had increased on the 4th-6^{th} and 8^{th}-10^{th} days compared to the other groups. These results revealed that the rats in HCHO group required more time in the test. In HCHO+CAPE group, however, on the 5^{th} and 7^{th}, the time used was significantly higher than that of the control group. Regardless of the time used during the injection days by the rats, the time used by the rats in HCHO group was significantly longer than that of the other groups (Figure 4).

Table I. The values of the time spent to find the cheese by the rats before the injections, n=7 per group.

Day	Control	HCHO	HCHO+CAPE	p
1	238.4±13.4	256.2±16.1	215.7±14.9	0.42
2	245.1±8.6	227.6±10.2	197.3±15.0	0.25
3	198.5±11.0	245.9±11.3	226.0±12.7	0.59
4	188.9±8.7	233.6±9.6	163.5±9.5	0.10
5	211.6±9.0	180.0±7.4	171.4±10.1	0.61
6	174.2±6.3	216.8±9.4	156.3±15.4	0.11
7	180.2±14.0	197.3±9.9	164.1±11.0	0.71
8	119.4±6.5	150.9±7.0	165.2±9.4	0.34
9	120.7±7.0	140.5±8.2	130.7±6.8	0.85
10	133.2±12.7	144.4±10.7	104.5±8.6	0.37

HCHO=formaldehyde, CAPE= caffeic acid phenethyl ester. Values are expressed as meanSD (two-way ANOVA for repeated measures).

Table II. The values of the time spent to find the cheese by three groups of rats during injection period, n=7 per group.

Day	Control	HCHO	HCHO+CAPE	p
1	128.7±10.1	141.3±13.3	104.8±8.8	0.75
2	130.2±9.3	120.3±8.2	112.2±12.1	0.90
3	106.0±5.0	148.2±10.2	132.5±13.0	0.71
4	102.6±8.2	144.4±9.7[a]	88.2±9.8	0.01
5	60.7±4.0	162.9±10.5[c]	114.1±14.1[a]	0.001
6	81.2±11.3	189.3±13.4[c]	99.0±8.6	0.001
7	72.8±7.2	159.2±9.4[b]	118.6±12.2[a]	0.001
8	58.4±5.4	168.5±8.8[c]	72.4±7.7	0.001
9	37.9±4.4	189.0±12.2[c]	66.1±8.0	0.001
10	48.4±8.9	183.2±14.7[c]	57.5±6.6	0.001

HCHO=formaldehyde, CAPE= caffeic acid phenethyl ester. Values are expressed as mean SD. [a] p<0.01 vs. other groups, [b] p<0.001 vs. control group, [c] p<0.001 vs. other groups (two-way ANOVA for repeated measures, post-hoc Tukey test).

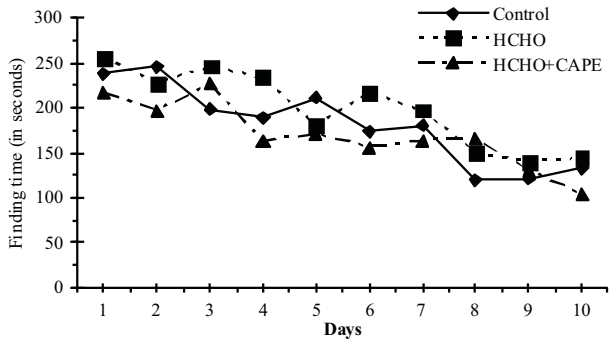

Figure 2. The time spent to find the cheese by the rats before the injections.

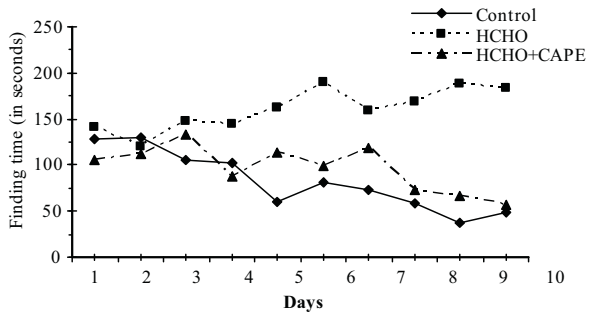

Figure 3. The time spent to find the cheese by the three groups of rats during injection period.

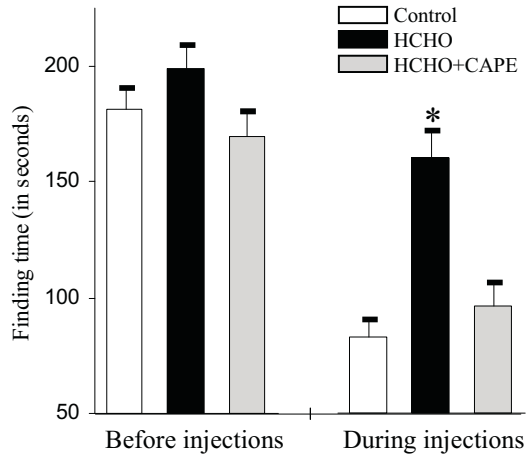

Figure 4. The mean values of the groups regardless of the day before and after the experiment and the comparison of the results. *When compared to the other groups p<0.001 (one-way ANOVA).

Discussion

Formaldehyde inflicts various harms on many systems. Its negative effects on the nervous system have been well documented in the epidemiological studies (14,23). Recently, there has been a trend for experimental studies. Kilburn et al. (15) in their study on histology technicians have shown that those who were exposed to formaldehyde through inhalation have suffered 3 times higher the difficulties of concentration and loss of short-term memory. Labyrinth test is one of the tests used in the evaluation of memory and learning. Pitten et al. (22) evaluated the neurotoxicity of HCHO inhalation with a labyrinth test. They exposed a group of rats to HCHO for 12 weeks and found that both the numbers of mistakes made while in the labyrinth and the time used to find the target had significantly increased (22). In another study using a swimming test to evaluate the effects of HCHO on learning and memory in rats, it has been reported that longer swimming period indicates negative effects of HCHO on the learning behavior and the memory of rats (1).

A previous study by Malek et al. (13) reported a statistically significant increase in the grooming acts of the rats inhaling HCHO twice a day every other day. Similar to the results of earlier reports, in our study, the time used by the rats in HCHO group to find the target (food) increased. The increased grooming activity by HCHO administered rats was considered a negative effect of HCHO on the behavior patterns of rats.

Boja et al. (24) detected a decreased total motor activity in the rats that inhaled 5 ppm HCHO in the acute phase. In addition, they observed neurochemical changes and attributed these changes to the increased levels of dopamine and serotonin in the rat hypothalamus. The neurotransmitters mentioned above act on the mental and cognitive functions. Bhatt and Panchal (25) studied the behavioral effects of HCHO administered both intraperitoneally and orally by inflicting electric shock and detected that intraperitoneally administered HCHO did not change the normal physiological activity of rats but led to memory disorders. In another study by Usanmaz et al. (4) on Balb/C mice, HCHO was found to increase the stimulation rate of central nervous system at low concentrations, but have depressing effects on the motor activity at increased concentrations. Intraperitoneal HCHO administration was reported to slow down the motor activity in another study (2). In the present study, the increased time used to find the food by rats is suggestive of slowed-down motor activity and development of memory disorders. Compatible with the results of Williams and Lees-Haley (26), HCHO can be said to have neurotoxic effects causing memory loss and learning difficulties.

The mechanism of the toxic effects of HCHO on the central nervous system is not clearly defined. However, upon passing the blood-brain barrier and communicating with the nervous system, HCHO is claimed to cause neurofilament increase (27). HCHO neurotoxicity has been reported to result from epoxide (reactive metabolic mediators arising from oxidation) production through which epoxides bind with axonal neurofilaments and render them nonfunctional, thus lead to intraaxonal swelling (28). Immediately after HCHO passes into the body, it oxidizes with formic acid in the liver and erythrocytes. Formic acid causes an increase in activity of cerebral lysosomal acid proteinase in cerebral hypoxia (29). Cerebral hypoxia affects the glial cells in the hippocampus (30), which are sensitive to hypoxia (31).

Central nervous system is highly sensitive to toxicity of reactive oxygen species due to its high oxygen consumption and poor antioxidant defense. Oxidative stress has been held responsible for neurodegeneration pathogenesis and many neurological diseases (32). CAPE, known with its antioxidant effect, is a phenolic agent of plant origin that increases both heme oxygenase activity and heme oxygenase-1 (HO-1) protein in astrocytes. HO-1 is a protein that protects the cells against oxidative stress (33). Montpied et al. (34) investigated the effects of CAPE against endotoxins in hippocampal cell culture and found that CAPE led to inhibition of nuclear factor B (NF-KB) activation in neuroinflammatory process. NF-KB is a very important factor in neuroinflammation and associated neuropathologies (34). At the same time, CAPE presents an apoptotic characteristic against tumor cells. This has been emphasized in cancerous cell culture studies (20,35). However, CAPE has shown a completely anti-apoptotic effect in low potassium induced cerebellar cell culture. This effect has been attributed to the ROS scavenger role of CAPE (36). Zararsiz et al. (2) reported that formaldehyde decreases SOD and GSH-Px activity in the prefrontal cortex, which is indicative of oxidative damage. In other words, HCHO leads to oxidative damage in the central nervous system. According to a recent published study, the lower count of apoptotic cells in HCHO+CAPE group than in HCHO group has suggested that CAPE has an antioxidant effect and protects cells (37). Thus, it is

assumed that CAPE exerts its protective effects in HCHO toxicity through its stimulating effects on the antioxidative defense system as well as its scavenging and/or antioxidant properties.

In conclusion, the findings obtained in the present study indicate that HCHO caused changes behaviors of the rats that were put through labyrinth test and altered their memory. CAPE, however, improved their memory possibly through its antioxidant and scavenging properties.

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