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Investigation of the dose-dependent effect of carminic acid on brain and peripheral tissues

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Abstract

Aim: In recent years, there has been an increase in the frequency of use of food additives and colorants to extend the life of food and to color it. Excessive consumption of these additives can lead to attention deficit hyperactivity disorder and learning disorder, especially in children. Uncontrolled additives whose dosage is not specified can cause an allergic reaction, cause damage to the neurological system and cause cancer. There are not many studies showing the dose-dependent effect of carminic acid. It was aimed to determine the dose-dependent changes of carminic acid, a food additive, in liver, kidney, blood and brain tissues (hippocampus, brain, brain stem) and to investigate the effects of these doses on hyperactivity behaviorally.

Materials and Methods: In our study, dose-dependent effects of 500 mg, 1500 mg and 3000 mg carminic acid (CA) used as a colorant in hippocampus, brain, brain stem, liver, kidney and blood samples were investigated.

Results: 32 Wistar albino male rats weighing 150-200 g were divided into 4 groups as control, CA-500, CA-1500 and CA-3000. The locomotor activities of the rats were evaluated in the open field test, and their anxiety behaviors were evaluated in the elevated plus maze test. Carminic acid levels in hippocampus, brain, brain stem, liver, kidney and blood were measured by high performance liquid chromatography (HPLC) method. Locomotor activity and anxiety behavior test results of CA-1500 and CA-3000 groups were increased compared to the control group. In the results of HPLC analysis, 500 mg, 1500 mg and 3000 mg doses administered to rats were not detectable in hippocampus, brain and brain stem tissues, while a dose-dependent increase was found in liver, kidney and blood samples ($p<0.05$).

Conclusion: It was concluded that since carminic acid could not cross the blood-brain barrier, it was not detected in hippocampus, brain and brain stem tissues, and increased dose-dependently in peripheral tissues such as liver, kidney and blood. Although it cannot be detected in the brain, it has been observed that hyperactivity, which may increase locomotor activity and anxiety, may lead to behavioral changes.

Introduction

Today, food additives are of special importance for many reasons such as reducing color loss during processing, increasing the existing color even more, and coloring colorless foods [1, 2]. These colorants can be in structures such as natural, synthetic, etc. Although natural colorants are physiologically compatible, their lack of stability reduces the preference for use [1]. Synthetic colorants, on the other hand, are produced by chemical synthesis but cannot be found naturally [1]. However, synthetic colorants generally have a more intense and permanent color than natural substances and are generally more stable [1]. Natural colorants are usually pigments produced by living organisms. Among them, carminic acid (E120) is a red glucocidal hydroxyanthrapurine that occurs naturally in some insect scales such as Dactylopius coccus and Polish cochineal [3]. Carminic Acid (CA), Carmine or Cochineal (E120), which is obtained from living organisms and is a natural colorant, is one of the most preferred among these colorants [2]. Approximately 150-180 tons of CA is produced each year, of which approximately 90% is produced by Peru [4]. CA imported to Turkey is primarily used in the textile and cosmetics industry. It is also used as a colorant in many products such as meat, salami, sausage, jam, ice cream, etc. in the food industry by using the E120 code with the permission of the European Commission. The dosage and duration of use

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of these additives, which are used as colorants, are important for health. The European Food Safety Authority (EFSA) has reported that CA is at the accepted daily intake level of 2.5 mg CA/kg/day. In a study, the acute oral toxicity of CA in mice (LD50 value of 6.250 mg/kg) was reported [4]. In another study, it was reported that 1,000 mg of carmine/kg/day CA did not cause toxicity in rats, and when a safety factor of 100 was taken into account, the " No-Observed-Adverse-Effect Level" (NOAEL) was reported to be 500 mg carmine/kg/day (Acceptable Daily Intake-ADI) [1]. According to research on additives, it has been reported that this substance causes an extreme allergic reaction in some people and also causes asthma and hyperactivity [5]. In some studies, it has been shown that food additives cause hyperactivity and learning disorders in children [5-7]. Since it is known that excessive use of food additives causes attention deficit hyperactivity disorder, especially in children, it is wondered whether carminic acid consumption will cause hyperactivity in children [4]. It has been reported that uncontrolled additives, whose dosage is not specified, may cause an allergic reaction, cause damage to the neurological system and cause cancer. There is no research showing what the effect and dosage of carmine are. Therefore, in our study; 1) determination of dose-dependent change of CA as a food additive in liver, kidney, blood and brain tissues (hippocampus, brain, brain stem), 2) behavioral investigation of the effects of these doses on hyperactivity are aimed.

Materials and Methods

Our study was carried out in Akdeniz University Experimental Animals Unit. Rats obtained from Akdeniz University Experimental Animals Application and Research Center with the approval of Akdeniz University Animal Experiments Local Ethics Committee (Decision No 39) were used in the study.

Experimental groups and protocol

32 Wistar albino male rats weighing 150-200 g were divided into 4 groups as control, CA-500, CA-1500 and CA-3000. The locomotor activities of the rats were evaluated in the open field test, and their anxiety behaviors were evaluated in the elevated plus maze test. Carminic acid levels in hippocampus, brain, brain stem, liver, kidney and blood were measured by HPLC method.

Experimental groups

Group 1: Control, 1 ml of distilled water was given by gavage (n=8),

Group 2: CA-500, 500 mg/kg/day CA was dissolved in 1ml distilled water and given by gavage (n=8),

Group 3: CA-1500, 1500 mg/kg/day CA was dissolved in 1ml distilled water and given by gavage (n=8),

Group 4: CA-3000, 3000 mg/kg/day CA was dissolved in 1ml distilled water and given by gavage (n=8).

Behavioral Experiments

Open Field Test

The open field test is used to evaluate locomotor activity. Open field experiments were performed in an 80x80x40 cm setup. Rats were placed in the central area of this area and their movements were recorded for 5 minutes using a camera system. The odor cues were eliminated by cleaning the open field setup with 70% ethanol for each rat. Locomotor activity was evaluated with the total distance (cm) and velocity (cm/sec) [8].

Elevated plus maze test

The elevated plus maze test is used to evaluate exploratory and anxiety-style behaviors of rodents. For the elevated plus maze test in rats, a 50x10 cm cross-section setup consisting of two open arms and two closed arms was used. The elevated plus maze assembly was cleaned with 70% ethanol and dried before each rat was placed in the assembly. Each rat was placed in the middle of the maze with its head facing the open arm and exploratory activity was monitored for 5 minutes. The time spent in open and closed arms and the numbers of entries were evaluated. Time spent in open arms (time in open arms/time in all arms x 100) and in closed arms (time in closed arms/time in all arms x 100) was calculated as a percentage. In addition, the percentages of open and closed arm entries (open arms/all arms or closed arms/(all arms) x 100) were evaluated. The presence of all four claws of the animal on one arm of the maze was defined as the entrance [9].

Biochemical method

Carminic acid analysis by HPLC (hippocampus, brain, brainstem, liver, kidney and blood) chemicals and reagents

Carmine and CA used as standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used in extraction and preparation of mobile phase, such as sodium hydroxide, sodium phosphate dibasic, and sodium phosphate monobasic were of analytical reagent grade and also supplied by Sigma–Aldrich. Water for all applications in our study was obtained from an Milli-Q ultra-pure water system (Millipore, Bedford, MA, USA) with resistivity equal to or higher than 18.2 MΩ cm.

Preparation of standard solutions

The stock solution (1000 lg ml^{-1}) of carmine was prepared by transferring 100 mg of carmine into a 100 ml beaker and adding 0.05 M NaOH solution to give a final volume of 100 ml. Calibration standard solutions at serial concentrations of carmine were obtained by mixing subsequent dilution (1–100 g/ml) with water.

High performance liquid chromatography

HPLC analysis was performed on an Agilent HPLC 1200 series (Santa Clara, CA, USA) coupled to a photodiode array detector. The LC system consisted of degasser, binary pump, autosampler, and column oven. An NovaPak C18 column (150 x 3.9 mm, 5 lm) purchased from Waters Corporation (Milford, MA, USA) was used for chromatographic separation. All separations were carried out isocratically at room temperature with a mobile phase consisting of methanol-phosphate buffer (pH 6.0) at ratios of 15:85 (v/v). The flow-rate was maintained at 0.8 ml min^{-1} and a 20 µl sample volume was injected into

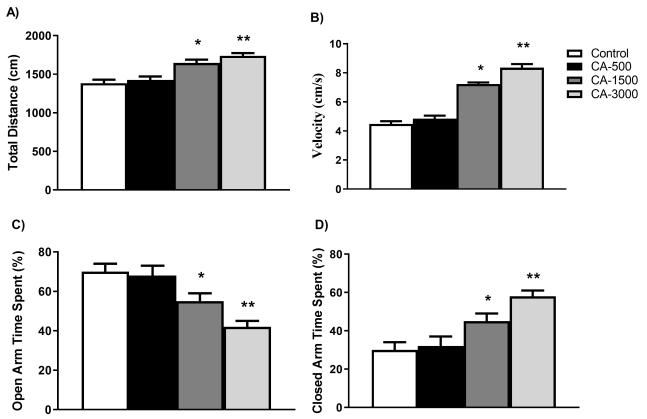

Figure 1. Open field test and elevated plus maze test findings of the experimental groups. A) Total distance (cm), B) Velocity (cm/sec), C) Time spent in open arm (%), D) Time spent in closed arm (%). * $p<0.05$ and **$p<0.001$ show the difference compared to the control group.

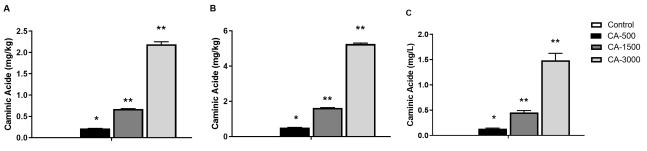

Figure 2. Carminic acid levels. A) Liver tissue carminic acid level (mg/kg), B) Kidney tissue carminic acid level (mg/kg), C) Blood tissue carminic acid level (mg/L). * $p<0.05$ and **$p<0.001$ show the difference compared to the control group.

HPLC. Carmine, eluted from the column, was monitored by photodiode array detector set at 281 nm. The absorption spectra of carmine were recorded between 200 and 500 nm. Peak identification was performed by comparing the retention time and absorption spectra of samples with the standard solution. Separation of carmine from carminic acid, a major component of cochineal extracts, was carried out in the same conditions as above [10].

Statistical analysis

The SPSS software package 20.0 program was used for all analyses. The results were given as mean±standard error (SEM). P values less than 0.05 were considered significant One-way ANOVA tests were used in the analysis of data with normal distribution in the evaluation made with the Shapiro Wilk test. The Tukey test was used for post-hoc analysis.

Results

When the open field test data were examined, it was found that the total distance (cm) and veleocity (cm/sec) values in the CA-1500 and CA-3000 groups increased statistically significantly compared to the control group (Figure 1A, 1B) ($p<0.05$). It was determined that there was an increase in the locomotor activity data of the CA-500 group compared to the control group, but this increase was not statistically significant. The increase in locomotor activity of the CA-3000 group was greater than the increase in the CA-1500 group compared to the control. In the elevated plus maze test, the CA-1500 and CA3000 groups showed a significant decrease in the rate of time spent in the open arm (%) compared to the control group, while an increase in the rate of time spent in the closed arm (%) was observed (Figure 1C, ID) ($p<0.05$). Compared to the control group, the CA-3000 group's anxiety resultr decreased more than the CA-1500 group's result.

CA levels were measured by HPLC method in hippocampus, brain, brain stem, liver, kidney and blood samples of rats. However, CA levels in the hippocampus, brain and brain stem tissues were not detectable. CA levels in the liver, kidney and blood seem to increase in a dose-dependent manner with 500 mg, 1500 mg and 3000 mg administered (Figure 2).

Discussion

Food additives, malnutrition, types of nutrition, eating habits, pharmacological effects, etc. have effects on behavior [6]. It is known that foods have negative effects on hyperactivity, attention, behavioral and learning disorders. It suggests that food additives (colorings, flavorings, and preservatives) may increase hyperactivity in children with behavioral problems. It can be accepted that nutrition regulation is more effective than drug treatment for children with behavioral problems such as hyperactivity [6]. Especially excessive consumption of foods containing food additives triggers these diseases even more. It is known that 6.250 mg/kg of CA causes low toxicity in rats, and that feeding 500 mg/kg of carminic acid for 90 days does not cause any toxicity in rats except for the unusual color change [4].

According to the European Food Safety Authority (EFSA), it has been reported that 2.5 mg/kg/day of CA does not cause toxicity. Considering the 100-fold safety factor, the "No-Observed-Side-Effect-Level" (NOAEL) was reported to be NOAEL 500 mg carmine/kg/day [1]. Therefore, in our study, it was investigated whether CA at 500 mg, 1500 mg and 3000 mg doses caused hyperactivity in rats and whether it was present in hippocampus, brain, brain stem, liver, kidney and blood samples. According to the study of Chung et al. (2001), carminic acid is known to cause an allergic reaction in humans [11]. CA has been reported to have the potential to promote the progression of capsular invasive carcinomas in a rat two-stage model of thyroid carcinogenesis [12].

In the results of our study, it was determined that 1500 mg and 3000 mg CA application caused a statistically significant increase in the distance and velocity results of the rats in the open field test and the number of entrances to the open arm in the raised plus maze test, while the elevated plus maze test and the time spent in the closed arm caused a decrease. It was observed that locomotor activity increased significantly and anxiolytic behaviors were exhibited, especially in the CA-3000 group, which was administered overdose. After administration of 500

mg, 1500 mg and 3000 mg CA was not detectable in the hippocampus, brain and brain stem tissues of rats. However, it was determined that after 500 mg, 1500 mg and 3000 mg carminic acid administration, it increased dose-dependently in liver, kidney and blood samples.

According to the results of our study, it was determined that carminic acid could not pass to the hippocampus, brain and brain stem, and the consumption of 500 mg carminic acid, which is an ADI, did not have any negative effects. However, 1500 mg and 3000 mg CA were observed to cause hyperactivity-like behaviors in rats. Especially in rats administered 3000 mg, locomotor activity was found to be greatly increased. In addition to all these, we can state that 1500 mg and 3000 mg of CA are far above the ADI, and it is difficult for children to consume such amounts of carminic acid. On the other hand, it was observed that the CA dose applied and the carminic acid levels detected in liver, kidney and blood samples were also correlated.

Conclusion

These results are the first to show that CA cannot pass into brain tissues, however, can be found in peripheral tissues such as liver, kidney and blood. In addition, this study also shows that carminic acid at an ADI does not have any negative effects on locomotor activity and anxiety behaviors. The results show that doses of 1,500 mg and 3,000 mg of CA above the ADI can cause hyperactivity in rats.

Ethics approval

Ethical approval was obtained for our study from the Akdeniz University Animal Experiments Local Ethics Committee (Decision No: 39).

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