Are there any remarkable effects of prenatal exposure to food colourings on neurobehaviour and learning process in rat offspring?

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Objective: Artificial food colourings and additives (AFCAs) have long been discussed to have adverse effects on cognition and behaviour in children. In this study, our aim was to assess the probable side effects of prenatal exposure to colouring food additives on neurobehaviour and spatial learning process.

Methods: We administered 'no observable adverse effect levels' (NOAELs) of common used AFCAs as a mixture (erythrosine, Ponceau 4R, Allura Red AC, Sunset yellow FCF, tartrazine, Amaranth, Brilliant Blue, Azorubine and Indigotine) to female rats before and during gestation and tested their effects on spatial working memory and behaviour in their offspring. Effects of AFCAs on spatial working memory were evaluated by Morris water maze, behavioural and locomotor effects by open-field and forced-swim tests.

Results: Prenatal exposure to commonly used AFCAs had no adverse effects on spatial working memory; however, assessment of interaction of sex and AFCAs on 'latency to locate the visible platform', which was used as a measure of motivation, showed a significant interaction ($P < 0.05$) on female rats. In addition, AFCAs caused an increase in anxiolytic like effect in the open-field test ($P < 0.05$) and an increase in mobility time ($P < 0.05$) in the forced-swim test. We also detected a significant interaction of sex and AFCAs on forced-swim test parameters ($P < 0.05$).

Discussion: These findings indicated that prenatal exposure to NOAELs of AFCAs resulted in implicit adverse effects that caused an increase in motility and a decrease in motivation and anxiety in offspring in sex-related manner.

Keywords: Artificial food colourings, Cognition, Forced swim test, Morris water maze, Open field test, Spatial learning

Introduction

Food colourings are used to impart, preserve, or enhance the colour of food and also shading of a food, including colour stabilizers, colour fixatives, colour-retention agents etc. Colours are usually classified as artificial (synthetic) or natural food colours, which indicates that they are, respectively, synthetically manufactured or obtained from natural sources.[1]

Artificial food colourings and additives (AFCAs) have long been suggested to adversely affect the learning and behaviour in children.[2] Firstly, Feingold hypothesized that hyperactivity was a child's adverse reaction to food additives, such as artificial sweeteners, artificial colours, and preservatives, that are present in numerous industrial foods and drinks.[3] Since then, the relationship between diet and hyperactivity, especially AFCAs effects on Attention-deficit hyperactivity disorder (ADHD) was widely investigated. A number of studies including double blinded, placebo-controlled trials have suggested a significant link between the long-term or repeated ingestion of synthetic food colours and behavioural hyperactivity.[4–7]

In addition to clinical studies, effects of various AFCAs on learning and behaviour have been studied in rats and mice by several researchers. Tanaka studied with different food colourings separately and focused on reproductive and neurobehavioural effects of them in rats. In most of these experimental studies, neurobehavioural procedures evaluated especially the neuromuscular developmental effects of AFCAs[8–14] in the first- and the second-generation

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offspring. In these studies, not all but some of the evaluated parameters like 'movement activity of exploratory behaviour' was significantly increased in a dose-related manner.[11,12] On the other hand, Dalal and Poddar showed that a single dose (10, 100, or 200 mg/kg, p.o.) of erythrosine (a common artificial dye) administration to young adult male rats reduced motor activity.[15] Gao *et al.* showed that the high and middle dose levels (350 and 700 mg/kg) of tartrazine (a common artificial dye) caused neurotoxicity and deficits in learning and memory in animals.[16]

In spite of these studies and assessments about AFCAs, the possibility of modification of behaviour and cognition and probable alteration in central nervous system metabolism by commonly used AFCAs remains a controversial and unresolved issue. We also have studied on this issue in our previous study and as the first step, we have administered a mixture of widely used synthetic AFCAs (erythrosine, Ponceau 4R, Allura Red AC, Sunset yellow FCF, tartrazine, Amaranth, Brilliant Blue FCF, Azorubine and Indigotine) with their acceptable daily intake (ADI) values, which are accepted safe for humans, to female rats and investigated their effects on cognition and behaviour in their offspring when they became adult. In the assessment of locomotor activity as well as exploration and anxiety, mixture of AFCAs showed a few significant effects which supported the hypothesis of increased locomotor activity and also we showed sex differences in AFCAs effects on neurobehaviour but we have found no effect of AFCAs on spatial learning and memory function.[17]

As we have found some implicit adverse effects in our previous study, we wanted to clarify whether those findings were dose related. The aim of this study was to assess the probable effects of prenatal exposure to synthetic food colourings on neurobehaviour and spatial learning process of offspring at the dose of 'no observable adverse effect level' (NOAEL), which is accepted safe for animals and a hundred times of the ADI value.

Materials and methods

Materials

Artificial food colourings and additives

The additives in the mixture of artificial food colours and the doses used in the study were as follows: erythrosine; 10 mg/kg/day,[18] Ponceau 4R; 70 mg/kg/day,[19] Allura Red AC; 700 mg/kg/day,[20] Sunset yellow FCF; 250 mg/kg/day,[21] tartrazine; 750 mg/kg/day,[22] Amaranth; 15 mg/kg/day,[23] Brilliant Blue FCF; 600 mg/kg/day,[24] Azorubine; 400 mg/kg/day,[25] and Indigotine; 500 mg/kg/day.[26] The doses used in this study were the NOAELs of these artificial food colours which were obtained from Joint Expert Committee on Food Additives monograph.[27] To prepare the mixture of artificial food colours, they were weighed separately and dissolved in water. The mixture was prepared weekly and stored at 4°C.

Animals

We acquired the animals from Animal Investigation Laboratory of Suleyman Demirel University. Animals were handled according to the recommendations for animal care and experimentation of the pertinent European Communities Council Directive (86/609/EEC), and all the procedures were approved by the Ethical Committee of the Medical Faculty of Suleyman Demirel University.

Thirty, Wistar Albino strain (aged 3–6 months, weighing 150–200 g), female rats were included in the study and categorized as control group ($n = 15$) and experiment group ($n = 15$). We had used 15 females as experiment group and 15 females as control group in order to provide genetic variability and prevent to use the animals which would be drawn from same litter. The rats were housed individually in solid-floored cages with wood flakes and kept in a temperature-controlled (23 ± 1°C) room. Rats were given *ad libitum* access to water and food.

The rats were weighed weekly and 1 ml of artificial food colour mixture and 1 ml of water per 100 g of their weight were administered daily by oral gavage to the experiment and the control group, respectively. Thus each rat was given at most 2 ml per day. Our main aim was to investigate the effects of prenatal exposure to food colourings on neurobehaviour and learning process in 1-month-old offspring which can be corresponded to human's early childhood period. Because the mixture contained nine colours and relatively high doses of the colours were planned to use, we tried to examine and overcome the administration problems like the digestion of the mixture, oral gavage administration, or the adaptation to the volume administered so we started the AFCA administration 1 week before gestation. At the end of 1-week administration, one male rat was placed in each cage and was allowed for 5 days to stay at the same cage to mate and the female rats became pregnant. During the pairing and the pregnancy period, administration of artificial food colour mixture to experiment group and water to control group was continued with the same dose, in the same way. After the partus of pregnant rats, offspring were allowed to stay with their mothers for a month for suckling. When the offspring were 1 month old, they were weaned in order to comprise the experiment and control groups which would be tested for neurobehavioural alterations in several tasks. Sixteen offspring (aged 1 month, weighing 50–100 g) of artificial food colour mixture administered rats were accepted as

experiment group (8 female + 8 male) and 16 offspring of water administered group (8 female + 8 male) were accepted as control group. Except a couple of offspring (a male and a female) which were drawn from a single litter, rest of the subjects of experiment group were drawn from different litters. The selection order of control group was the same as the experiment group; the offspring were drawn from different litters, only one male and one female were drawn from a single litter. So only one subject of each sex is used in the statistical analysis from each litter.

Following the generation of the groups, the rats were transferred to the laboratory where the trainings of spatial learning and memory, as well as locomotor, anxiety, and depression tests would be done and waited for a week for adaptation to the laboratory. The colony room was maintained under an automatically regulated 12:12-hour reverse light/dark cycle (lights off at 08:00 a.m.) and testing occurred during the dark phase which was their most active period.[28] Both of the groups were trained and tested in the Morris water maze (MWM), afterwards tested in the open field test and the forced swim test to assess the effects of AFCAs on spatial learning, behaviour and locomotor activity.

Behavioural tests

Morris water maze

MWM is a task that evaluates spatial learning and memory, a measure of cognitive function. The tank was placed in a dimly lit, soundproof test room with various visual cues. This task uses a round pool of water in which a platform is submerged beneath the surface. When placed in the maze the animal's task is to find the hidden platform. The MWM consisted of a circular pool, 150 cm in diameter and 80 cm in height, with the interior painted white. The temperature of the water was checked daily by a thermometer and adjusted to 22 ± 2°C and was made opaque by the addition of nontoxic dark yellow paint. The pool was surrounded by four halogen lamps (300 W with adjustable power range), which were directed to the walls that surrounded the pool. Visible extra-maze cues including a picture on the wall, a chair, and a coloured box were left at fixed positions around the water maze.

Experimental procedure

Each trial was tracked by using an overhead camera (Sony SSC-DC398P, Sony Corporation, Shinagawa, Japan) interfaced with a computer that recorded the training phase and probe trial test for all the runs in the maze. The tracking program we used was Smart Version 2.5 (Smart Video-Tracking, Panlab, Barcelona, Spain).

The maze was divided into four equally sized virtual quadrants, designated as zones one to four. The platform remained in a fixed location for all runs and the target quadrant was the fourth quadrant.

Throughout the experiment, animals were handled before the first trial of each day and then were released once from each of the four quadrants facing the centre of the pool. Daily training consisted of five trials in which the rat was placed in the water from four random starting positions (1, 2, 3, and 4) and the latency of escaping onto the platform was recorded. Starting locations were equally spaced around the perimeter of the pool. This was conducted for 4 consecutive days. In a protocol modified from Morris, acquisition of place learning using spatial cues and navigational strategy was done on days 1–4 and then the test for memory called 'probe trial' was performed on day 5.[29]

On the first day of testing, animals were allowed to swim in the pool for 60 seconds. If a rat could not find the hidden platform, it was placed on the platform for 30 seconds to introduce the platform and show that the platform is the mean of escape from the water. On day 2, if a rat could not find the platform, it was placed on the platform by the experimenter and were left on the platform for 15 seconds and then removed to their home cages by the experimenter. There were five trials per day, with an intertrial interval of approximately 20 minutes for each rat. After trials, subjects were dried with a towel and warmed under a 40-W soft white bulb (Osram, OSRAM AG, Munich, Germany) before being returned to the home cages. At the end of the fourth day, each rat had been trained 20 times totally and the acquisition period had been completed.

On day 5, the platform was removed and the rats were released from the three other quadrants which did not contain the hidden platform before. At this probe trial test, the time spent in the target quadrant where the platform had been during acquisition period was recorded. The percentage of swimming in the quadrant of the former platform was calculated as a measurement of spatial memory. At day 1 and following days if the time taken was greater than 60 seconds, it was recorded as 70 seconds. On day 6, visible platform procedure was applied. This is the cued version of the Morris water escape task which is used to eliminate the direct or indirect effects of the food colourings such as decreased motivational factors, increased anxiety of animals in the water and therefore decreased the desire to escape. Each rat was released from the fourth quadrant all the time and visible platform was carried to the different quadrants for each trial except the fourth quadrant. Again escape latency to the visible platform was recorded.[29]

Open-field test (OFT)

The open-field maze (arena) was used to analyze spontaneous exploratory and locomotor activity and

anxiety-related behaviours.[30] The apparatus consisted of a square arena (100×100 cm). The floor was marked into equal 16 segments and arena was divided into three virtual segments as 'Outer Zone', 'Inner Zone', and 'Centre'. Four halogen lights were directed to the walls surrounding the apparatus, so diffuse overhead illuminations were provided. The animals were tested in a quiet room and the locomotor activity over a 5-minute period was recorded using a ceiling-mounted videocamera (Sony SSC-DC398P, Sony Corporation, Shinagawa, Japan) and transferred the data to the computer using the Smart Version 2.5 program (Smart Video-Tracking).

Each animal was tested individually in the open-field maze. Each animal was allowed to explore the maze for 5 minutes and behaviours were scored. The behaviours scored were as follows: the number of lines crossed with at least three paws of the rat, number of rearing, number of walling, the time spent at the edge of the apparatus (outer zone), the time spent at inner zone and center arena seperately, the travelled distance in three different segments (outer, inner, centre), velocity, total length travelled, number of center square activity, and number of defecation was recorded by a rater who was blind to the animals' housing conditions. Line crossing (the number of line crosses) is a form of locomotor behaviour – horizontal locomotor activity. Rearing is defined as the animal standing upright on its hind legs. Walling is defined as the animal standing upright on its hind legs and front legs on the maze's walls. Walling and rearing are the vertical activities that especially indicate anxiolytic like effects. Generally higher frequency of line crosses, rearing, walling, central square frequency, centre square duration were indicating increased locomotion and exploration and low anxiety as well as higher frequency of edge duration and number of defecation were indicating lower exploratory behaviour and higher anxiety.[31]

Forced swim test (FST)

When rats are forced to swim in an inescapable situation, they tend to become immobile after initial vigorous activity. This immobility has been described as a symptom of a behavioural despair.[32] The test was conducted using a modification of the method of Porsolt. Briefly, rats were individually placed in a 45-cm high and 20 cm diameter transparent cylindrical apparatus (Any Maze, Wood Dale, USA) containing 40 cm of water with ambient temperature (23°C), so that the rat's hindlimbs could not reach the tank's floor. Rats were allowed to swim for 6 minutes and their activity was tracked.[33] The videotaped behaviour was subsequently analyzed by a rater who was blind to the animals' housing conditions. Duration of immobility was defined as when the rat was stationary and only made the minimal movements necessary to stay afloat, and mobility was defined as swimming, jumping, rearing, sniffing, or diving which were considered active, escape directed behaviour. All of these movements were tracked using an overhead camera (Sony SSC-DC398P, Sony Corporation, Shinagawa, Japan) interfaced with a computer which was using the Smart Version 2.5 program (Smart Video-Tracking).

Statistical analysis

The statistical analyses were carried out using the SPSS 15 for Windows program. The latency data were collected over 4 days with five trials being run every day. The five trials were averaged per day; therefore, day was the only within-subject variable and group was the between-subject variable. We assessed data for homogeneity by Levene test; as the data were homogeneous, a univariate repeated-measure analysis of variance (ANOVA) was used for intragroup comparison of performances of each group from days 1 to 4. The Greenhouse-Geisser correction was used. A P value of less than 0.05 was considered statistically significant. By this analysis, the effect of time (day) for each group was assessed. For the significant values we used t-tests with Bonferroni correction as *post hoc* test and a P value of less than 0.01 was considered statistically significant. The results were given as mean ± SD (standard deviation).

In between-group comparisons of MWM, FST, and OFT data, we assessed data for homogeneity by Levene test; as the data were homogeneous, we used Independent Samples t-test for comparisons. The parameters compared between groups were as follows: 'latency to locate the hidden platform' on each day, 'time spent at the target quadrant' of probe trial (day 5), 'latency to locate the visible platform' (day 6) and 'swim speeds' (training and test days), 'the number of lines crossed', 'number of rearing', 'number of walling', 'the time spent at the outer zone, inner zone and center arena', 'the distance travelled in the 3 different zones', 'velocity', 'total length travelled' and 'number of center square activity' (data of OFT), 'mobility and immobility periods', and 'speed' (data of FST). A P value of less than 0.05 was considered statistically significant.

We also compare groups as female and male groups for the data of MWM, FST, and OFT by Independent Samples t-test. P value of less than 0.01 was considered statistically significant. The results were given as mean ± SD.

To assess the effect of the interaction of sex and AFCAs on 'latency to locate the hidden platform' over days 1–4, we used multivariate ANOVA. Univariate ANOVA (two-way ANOVA) was used for

each test parameter for the rest of the data, and P value of less than 0.05 was considered statistically significant.

Results

Morris water maze performance

Latency to locate the hidden platform

Analysis of day-to-day latencies was performed in order to determine the time (day) effect for each group. Greenhouse-Geisser correction was used to evaluate within-subjects effects ($df = 2.12$, $F = 105.42$, $P < 0.0005$) which was followed by Bonferroni correction. Day-to-day intragroup comparison of MWM performances of the rats were significantly improved by days 2, 3, and 4 when compared with day 1 performance in both groups (control group: days 1–2 ($t = 7.73$, $df = 15$, $P < 0.0005$), days 1–3 ($t = 7.53$, $df = 15$, $P < 0.0005$), days 1–4 ($t = 7.97$, $df = 15$, $P < 0.0005$), and experiment group: days 1–2 ($t = 10.75$, $df = 15$, $P < 0.0005$), days 1–3 ($t = 8.36$, $df = 15$, $P < 0.0005$), days 1–4 ($t = 10.56$, $df = 15$, $P < 0.0005$)). This significant improvement indicates that all of the rats had a prominent and sustained enhancement in learning over the 4-day training period (Fig. 1A). No significant difference was found between control and experiment groups about spatial learning times over days 1–4 (day 1: $t = -0.36$, $df = 30$, $P = 0.72$; day 2: $t = 1.23$, $df = 30$, $P = 0.23$; day 3: $t = -0.20$, $df = 30$, $P = 0.84$; day 4: $t = 1.18$, $df = 30$, $P = 0.25$) (Fig. 1B). Comparison of groups as female and male groups showed no significance about spatial learning times over days 1–4 either (day 1: $t = 1.69$, $df = 30$, $P = 0.1$; day 2: $t = 0.91$, $df = 30$, $P = 0.36$; day 3: $t = 1.42$, $df = 30$, $P = 0.16$; day 4: $t = 1.57$, $df = 30$, $P = 0.13$). In addition no interaction of sex and AFCA administration was determined on 'latency to locate the hidden platform' over days 1–4 (day 1 ($F(1,28) = 0.19$, $P = 0.66$), day 2 ($F(1,28) = 0.03$, $P = 0.87$), day 3 ($F(1,28) = 0.0001$, $P = 0.1$, day 4 ($F(1,28) = 1.61$, $P = 0.22$)).

Time spent in the target quadrant

In the probe trial, analysis of groups' data by independent samples T-test showed that there was no significant difference between control and experiment groups about the 'time spent in the target quadrant' ($t = 1.34$, $df = 30$, $P = 0.19$) (Table 1). Comparison of groups as females and males showed no significance either ($t = -0.95$, $df = 30$, $P = 0.35$). Analyses of data showed no interaction of sex and AFCA administration on 'time spent in the target quadrant' ($F(1,28) = 1.92$, $P = 0.18$)).

Latency to locate the visible platform

Comparison of the data: 'latency to locate the visible platform' showed no significant difference between control and experiment groups ($t = -0.85$, $df = 30$, $P = 0.40$) (Table 1). Comparison of groups as females and males showed no significance ($t = 0.83$, $df = 30$, $P = 0.42$). However, assessment of interaction of sex and AFCA administration on this behaviour showed a statistically significant interaction at the $P = 0.04$ level ($F(1,28) = 4.78$). Simple main effects analysis showed that the females in experiment group spent significantly more time to locate the visible platform than males ($F(1,28) = 4.69$, $P = 0.04$) but there was no statistically significant mean difference between females and males on the 'time spent to

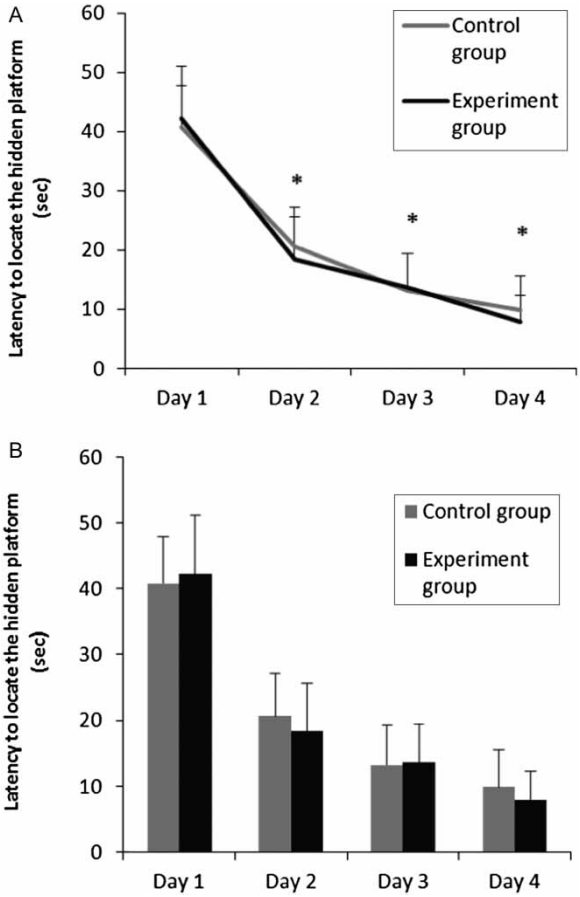

Figure 1 (A) Effects of prenatal exposure to AFCAs on spatial working memory performance: latency to locate the hidden platform (intragroup comparison). 'Repeated measures ANOVA' was used for intragroup comparisons ($F(1,28) = 105.42$, $P < 0.0005$), followed by T test. Data are expressed as mean ± SD. Day-to-day intragroup comparison of the rats in MWM was significantly improved by days 2, 3, and 4 as compared to day 1 in both groups. So, all of the rats showed progressively enhanced learning over the 4-day test period. *Significant difference when compared with day 1 performance of each group ($P < 0.0005$). (B) Effects of prenatal exposure to AFCAs on spatial working memory performance: latency to locate the hidden platform (Intergroup comparison). Day-to-day comparison of the data between groups was performed by using Independent samples t-test. Data are expressed as mean ± SD ($n = 16$). The x-axis presents the training period. No significant difference was found between groups about spatial learning times over days 1–4. Examination of these data presented us that rate of learning in experiment group showed no difference when compared to the control group.

Table 1 Effects of prenatal exposure to AFCAs on spatial working memory performance*

	Probe trial (sec)	Visible platform (s)	Distance travelled (cm)	Swim speed (cm/s)
Control group ($n = 16$)	29.23 ± 7.41	9.46 ± 5.58	1943.07 ± 393.19	32.82 ± 6.30
Experiment group ($n = 16$)	26.35 ± 6.85	12.15 ± 11.39	2112.07 ± 288.98	35.30 ± 4.82

*Statistical analysis of these data were performed by using Independent samples *t*-test. Data are expressed as mean ± SD. Analysis of groups' data of the probe trial showed that there was no significant difference between groups about the 'time spent in the target quadrant', 'latency to locate the visible platform', 'distance travelled', 'swim speeds'.

locate the visible platform' in control group ($F(1,28) = 0.86$, $P = 0.36$) (Fig. 2). In consideration of these findings, we can indicate that AFCA administration effected 'time spent to locate the visible platform' behaviour especially in females, which was used as a measure of decreased motivation.

Swim speeds

Swim speeds were calculated by sampling path lengths (cm) and measuring the time (seconds) spent to locate the hidden platform for each rat. To evaluate the swim speeds, data of training days, data of probe trial (day 5) and visible platform procedure (day 6) were used. Comparison of the data: 'distance travelled' showed no statistically significant difference between groups ($t = -1.39$, df = 30, $P = 0.18$). Either comparison of the swim speeds between control and experiment groups showed significant difference ($t = -1.25$, df = 30, $P = 0.22$) nor comparison of swim speeds between females and males showed significant difference ($t = -1.23$, df = 30, $P = 0.23$). Also no interaction of sex and AFCA administration ($F(1,28) = 0.04$, $P = 0.85$) was detected on this parameter. The swim speeds remained very stable for all of the rats over all test days (Table 1).

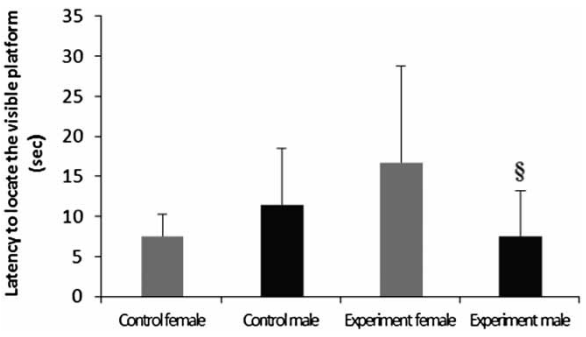

Figure 2 Interaction of prenatal exposure to AFCAs and sex on the 'latency to locate the visible platform'. Interaction of sex and AFCAs on evaluated parameter was assessed by two-way ANOVA. Assessment of interaction of sex and AFCA administration on this behaviour showed a statistically significant interaction ($F(1,28) = 4.78$, $P = 0.04$). Simple main effects analysis showed that the females in the experiment group spent significantly more time to locate the visible platform than males ($F(1,28) = 4.69$, $P = 0.04$). In consideration of these findings, we can indicate that AFCA administration affected 'time spent to locate the visible platform' behaviour especially in females, which was used as a measure of decreased motivation. §Significant difference when compared with female experiment group ($P < 0.05$).

Open-field test performance

Parameters assessed in open-field test and their comparison between control and experiment groups by independent samples *T*-test were as follows: number of line crosses ($t = -0.61$, df = 30, $P = 0.54$), number of rearing ($t = -1.3$, df = 30, $P = 0.2$), number of walling ($t = -2.73$, df = 30, $P = 0.01$), centre crosses ($t = -0.89$, df = 30, $P = 0.38$), number of defecation ($t = 0.20$, df = 30, $P = 0.84$), distance in outer ($t = -0.25$, df = 30, $P = 0.81$), inner ($t = -1.16$, df = 30, $P = 0.26$) and center zones ($t = -0.04$, df = 30, $P = 0.97$), time spent in outer ($t = 1.43$, df = 30, $P = 0.16$), inner ($t = -1.90$, df = 30, $P = 0.07$) and centre zones ($t = 0.05$, df = 30, $P = 0.96$), and movement speed during the test ($t = -0.43$, df = 30, $P = 0.67$).

Comparison of the same parameters between female and male groups by independent samples *T*-test was as follows: number of line crosses ($t = 3.25$, df = 30, $P = 0.003$), number of rearing ($t = 1.20$, df = 30, $P = 0.24$), number of walling ($t = 2.41$, df = 30, $P = 0.22$), centre crosses ($t = 0.41$, df = 30, $P = 0.14$), number of defecation ($t = 0.41$, df = 30, $P = 0.68$), distance in outer ($t = 2.98$, df = 30, $P = 0.01$), inner ($t = 1.66$, df = 30, $P = 0.19$), and centre zones ($t = 1.31$, df = 30, $P = 0.2$), time spent in outer ($t = -1.32$, df = 30, $P = 0.19$), inner ($t = 1.03$, df = 30, $P = 0.31$), and centre zones ($t = 0.9$, df = 30, $P = 0.38$), and movement speed during the test ($t = 0.62$, df = 30, $P = 0.01$).

The assessment of interaction of sex and AFCA administration on these parameters were as follows: (number of line crosses: $F(1,28) = 0.36$, $P = 0.55$, number of rearing: $F(1,28) = 0.002$, $P = 0.96$, number of walling: $F(1,28) = 0.08$, $P = 0.78$, centre crosses: $F(1,28) = 0.02$, $P = 0.88$, number of defecation: $F(1,28) = 2.06$, $P = 0.16$, distance in outer: $F(1,28) = 0.17$, $P = 0.69$, distance in inner: $F(1,28) = 0.09$, $P = 0.76$ and distance in center zone: $F(1,28) = 0.48$, $P = 0.49$, time spent in outer: $F(1,28) = 0.29$, $P = 0.59$, inner: $F(1,28) = 0.01$, $P = 0.90$ and centrr zones: $F(1,28) = 0.07$, $P = 0.78$, and movement speed during the test ($F(1,28) = 0.07$, $P = 0.79$) (Table 2).

The 'number of walling' behaviour was significantly higher in experiment group when compared to control group which is a vertical movement suggested as

Table 2 Effects of prenatal exposure to AFCAs on the evaluated parameters in the Open-Field Test

Groups	No. of line crosses	No. of walling	No. of rearing	No. of defecation	Distance in outer zone	Distance in inner zone	Distance in center zone	Time in outer zone	Time in inner zone	Time in center zone	Velocity
Control group ($n = 16$)	51.38 ± 29.73	10.94 ± 7.16	3.94 ± 3.95	2.94 ± 1.914	1751.02 ± 740.29	80.40 ± 76.99	24.29 ± 25.53	288.13 ± 8.54	4.33 ± 4.64	1.51 ± 2.30	6.38 ± 2.74
Experiment group ($n = 16$)	57.00 ± 21.55	18.81 ± 9.04*	7.19 ± 9.101	3.06 ± 1.526	1807.18 ± 522.98	123.21 ± 126.51	24.79 ± 38.68	282.66 ± 12.63	9.41 ± 9.68	1.47 ± 2.32	6.76 ± 2.17

*Significant difference when compared with control group ($P < 0.05$). Statistical analysis of these data was performed by using Independent samples *t*-test and interaction of sex and AFCAs on evaluated parameters were performed by two-way ANOVA. Data are given as mean ± SD. Effect of AFCAs on 'number of walling' behaviour was significantly increased in experiment group as compared to control group ($t = -2.73$, df = 30, $P = 0.01$) and assessment of interaction of sex and AFCAs on this parameter showed no significance ($F(1,28) = 0.08$, $P = 0.78$).

anxiolytic like effect. On the other hand, comparison of groups as female and male showed that the parameters; 'line crosses', 'distance in outer zone', and 'movement speed' were significantly different. Assessment of interaction of sex and AFCAs showed that there were no significant effect in females and males neither in control nor in experiment groups. Administration of AFCAs showed its effect on this behaviour similarly in female and male sex.

Forced swim test performance

Comparisons of mobility (active behaviour) ($t = -3.13$, df = 30, $P = 0.004$) and immobility periods ($t = 4.45$, df = 30, $P < 0.0005$) of the control and expeiment groups showed statistically significant difference between groups as experiment group was significantly more active when compared to control group (Fig. 3). When the groups were compared as female and male groups significant difference was found in mobility period ($t = 2.66$, df = 30, $P = 0.01$) as females had significantly longer mobility period compared to male group. The assessment of interaction of AFCA administration and sex on these parameters also showed significance on both of the parameters (mobility period: $F(1,28) = 8.20$, $P = 0.008$, immobility period: $F(1,28) = 13.61$, $P = 0.001$). In control group, mobility period was significantly shorter in males when compared to females ($F(1,28) = 19.96$, $P < 0.0005$). However, in experiment group there was no significant difference between males and females ($F(1,28) = 0.12$, $P = 0.73$) about mobility period (Fig. 4).

Discussion

The findings of this study showed that prenatal exposure to AFCAs had no obvious adverse effect

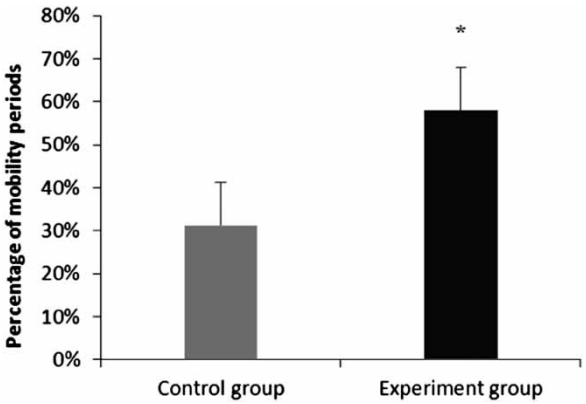

Figure 3 Effects of prenatal exposure to AFCAs on 'Mobility period' in the Forced Swim Test. Comparison of the data between groups was performed by using Independent samples *t*-test. Data are expressed as percentage of means of the mobility periods for groups. Analyses of the data showed that experiment group was significantly more active than control group ($t = -3.13$, df = 30, $P = 0.004$). *Significant difference when compared with control group ($P < 0.05$).

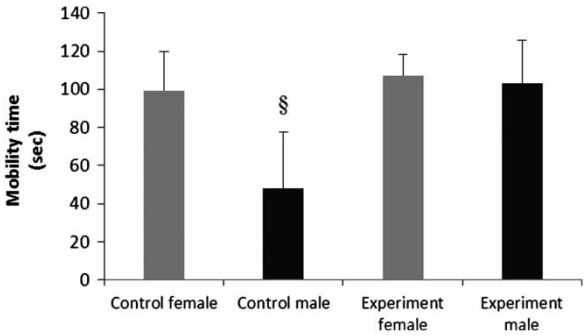

Figure 4 Interaction of prenatal exposure to AFCAs and sex on the forced swim test performances. Interaction of AFCAs and sex on evaluated parameter was assessed by two-way ANOVA. The assessment of interaction of AFCA administration and sex on mobility period showed significance ($F(1,28) = 8.20$, $P = 0.008$). In control group, mobility period was significantly shorter in males when compared to females (($F(1,28) = 19.96$, $P < 0.0005$)). However in experiment group there was no significant difference between males and females about mobility period (($F(1,28) = 0.12$, $P = 0.73$)). Examination of these analyses represented us that AFCAs significantly increased locomotor activity of male rats and males reached to females' performances which resulted in significant increase in mobility period in experiment group. §Significant difference when compared with female control group about mobility period ($P < 0.05$).

on hippocampus-dependent spatial learning and memory in offspring. The learning rates of the groups and the test of previously learned information showed no difference between groups, but an interaction of sex and AFCA administration was detected on the 'latency to locate the visible platform' which was used as a measure of motivation. Especially the females in experiment group were effected, so it should be suggested that AFCAs might cause a decrease in motivation in female sex. AFCAs also caused a significant effect on the 'number of walling' parameter which is mostly indicated as an anxiolytic like effect (increased exploration/ decreased anxiety). The obvious effect of AFCAs was detected in the forced swim test so that experiment group was significantly more active. In addition, interaction of sex and AFCA administration was determined to have effect on motility in the FST. In control group, male rats had significantly more inactive behaviour compared to female rats but in experiment group, male and female rats showed no difference. These findings may be suggested as supporters of hyperactivity like behaviour which has been suggested by several researches before.[6,7,12,13,34,35]

We also have studied on this issue in our previous study, with the same design except the doses, as we have administered the ADI values. We showed a few adverse effects of AFCAs which supported the hypothesis of increased locomotor activity and also we showed that AFCAs' effects on neurobehaviour were different from each other in both sexes.[17] In several animal studies, different kind of food dyes (sulfanilic acid, erytrosine, tartrazine) were administered individually or as a mixture and were shown to increase locomotor activity and impair learning function in different tasks like T maze, OFT.[16,36,37] While our findings were similar with these studies about locomotor activity, we have found no difference in learning functions. On the other hand, we have to mention the fact that our studies' experimental design was totally different from those studies. We had used a mixture of AFCAs in mothers during pregnancy and assessed the effects of AFCAs on neurobehaviour in offspring but in those studies, the dye had been administered directly to the offspring/ adults and effect of different levels of the dye were assessed in particular sex.

Tanaka studied on neurobehavioural effects of several food dyes (erythrosine, tartrazine, Sunset yellow FCF, Ponceau 4R, Amaranth, and Allura red AC) which were administered separately to female rats and determined their effects on several neurobehavioural parameters in F0–F3 generations with several tasks. These researches showed that AFCAs caused adverse effects on several neurobehavioural parameters related to movement activity of exploratory behaviour. These effects seemed to be dose-related since they were seen with the middle and high doses which were greater than human ADI. In addition, different dyes and different doses of the same dye caused different effects in male and female mice.[8–14] Our study was substantially in accordance with Tanaka's studies about experimental design and results, as well. The differences of our study from Tanaka's studies were namely, administration of the food colourings not one by one in each study with different doses, experimental tasks, and parameters used for assessing neurobehavioural effects.

Among the animal studies in which AFCAs' effects on neurobehaviour was assessed, the administration of food dyes appeared to increase spontaneous locomotor activity and effect several parameters of cognitive function. However, the tasks and parameters used in the assessment of neurobevioral changes differed from each other and displayed different degrees of complexity. Different criterions were used for evaluation, so different parameters were reported as significantly altered among studies. As a result, while our findings were consonant with most of the mentioned animal studies, some of them conflicted with ours. The contradictory findings could be explained by the differences in the design of the studies. In most of the studies, the fact that AFCAs had been administered to rats/mice and their effects were evaluated simultaneously was the different aspect from ours.

Although the purpose of these animal studies was providing additional data to clarify the probable side effects of AFCAs on behaviour and memory, we see

that the researches carried on human and animals do not overlap completely so we have to mention that the selected tasks that were used to evaluate the neurobehavioural effects in experimental studies cannot be directly used to suggest an association with ADHD, but they would provide the signs of increased locomotor activity and difficulties in learning function.

Conclusion

In this study, mixture of AFCAs which were used with NOEHLs during pregnancy had no adverse effect on spatial working memory, but decrease motivation in females, increase motility in males and increase anxiolytic like behaviour in both sexes. Thus, we can suggest that AFCAs would have different degrees of effects on male and female sexes which caused different neurobehavioural consequences.

The limitation of this study was that we did not assess whether one of the colouring or the interaction of two or more colourings in the mixture were responsible for the present findings. We did not also investigate the underlying mechanisms of these adverse effects of AFCAs on neurobehaviour. The difference in effects of AFCAs on male and female sexes led us think that the AFCAs may effect different hormones and/or neurotransmitter pathways in both sexes. Effects of AFCAs on expression of learning- and memory-related neurotransmitters should also be assessed in the future studies. It would also be useful to analyse the effects of the ingredients of the mixture separately on neurobehaviour to find out which colouring was responsible for the findings in the future studies.

Conflict of interest

All the authors disclose that there was not any financial, personal, or any relationships with other people or organizations within 3 years of beginning the work submitted that could inappropriately influence the work submitted.

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